# Genotype-phenotype studies in rare chromosome aberrations

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# Genotype-phenotype studies in rare chromosome aberrations

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*Voor mijn ouders*

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## ABBREVIATIONS

| | |
|---|---|
| AF | Amniotic Fluid |
| BAC | Bacterial Artificial Chromosome |
| BAEP | Brainstem Auditory Evoked Potentials |
| BAHA | Bone Anchored Hearing Aid |
| BERA | Brainstem Evoked Response Audiometry |
| bp | base pair |
| BP | breakpoint |
| CAA | Congenital Aural Atresia |
| CHD | Congenital Heart Disease |
| CGH | Microarray-based Comparative Genomic Hybridization |
| CHARGE | Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities and deafness |
| CNV | Copy Number Variation |
| CT | Computer Tomography |
| DECIPHER | DatabasE of Chromosome Imbalance and Phenotype in Humans Using Ensembl Resources |
| DNA | Desoxyribo Nucleic Acid |
| DSM | Dense Surface Models |
| ECARUCA | European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations |
| EEG | ElectroEncephaloGraphy |
| FISH | Fluorescent *In Situ* Hybridization |
| fMRI | functional Magnetic Resonance Imaging |
| GH | Growth Hormone |
| ID | Intellectual Disability |
| IUGR | Intra Uterine Growth Retardation |
| IQ | Intelligent Quotient |
| kb | kilobase (thousand base pairs) |
| MACRs | malformation-associated chromosome regions |
| Mb | Megabase (million base pairs) |
| MCA | Multiple Congenital Abnormalities |
| MLPA | Multiplex Ligation-dependent Probe Amplification |

| | |
|---|---|
| MR | Mental Retardation |
| MRI | Magnetic Resonance Imaging |
| NT | Nuchal Translucency |
| OMIM | Online Mendelian Inheritance in Man |
| PCR | Polymerase Chain Reaction |
| PHS | Pitt Hopkins Syndrome |
| QF-PCR | Quantitative Fluorescence Polymerase Chain Reaction |
| qPCR | quantitative Polymerase Chain Reaction |
| SD | Standard Deviation |
| SMS | Smith Magenis Syndrome |
| SNP | Single Nucleotide Polymorphism |
| TOP | Termination Of Pregnancy |
| TSH | Thyroid Stimulating Hormone |
| UCSC | University of California, Santa Cruz |
| WBS | Williams-Beuren Syndrome |
| WHS | Wolf-Hirschhorn Syndrome |
| WHSCR-1 | WHS critical region 1 |
| WHSCR-2 | WHS critical region 2 |

chapter 1

# General introduction and outline of this thesis

Based on:

Ilse Feenstra, Han G. Brunner and Conny M.A. van Ravenswaaij-Arts
Cytogenetic genotype-phenotype studies: Improving genotyping, phenotyping and data storage
Cytogenet Genome Res 115:231–239 (2006)

## From microscope to microarray

The genetic information is carried by DNA, which is packaged in chromosomes, tread-like structures located in the nucleus of the cell. Each chromosome is made up of DNA tightly coiled many times around histones that support its structure. The number and appearance of chromosomes in the nucleus of a eukaryotic cell is referred to as the karyotype. In 1956, the total number of chromosomes per cell in humans was visualized by microscope and determined to be 46, consisting of 22 pairs of autosomes and a single pair of sex chromosomes. [1] A few years later the underlying genetic cause of Down syndrome was revealed to be an extra chromosome 21. [2] It appeared to be that chromosomal rearrangements are an important cause of distinctive and recognizable clinical phenotypes. Subsequently, other numerical chromosomal aberrations have been detected in patients presenting with an overlapping phenotype, including Patau syndrome (trisomy 13) [3], Edwards syndrome (trisomy 18) [4], Turner syndrome (45,X) [5] and Klinefelter syndrome (47,XXY). [6]

With the implementation of chromosome banding techniques, not only numerical but also structural rearrangements and partial chromosome aberrations could be identified. [7-9] This has led to the identification and categorization of numerous unbalanced chromosome aberrations in individuals with intellectual disability and/or congenital anomalies such as deletions and duplications, as well as inversions and translocations. [10] However, small chromosome aberrations of less than 5 Mb cannot be detected by standard karyotyping.

The introduction of fluorescence *in situ* hybridization (FISH) techniques and multiplex ligation-dependent probe amplification (MLPA) allowed for the detection of small, submicroscopic cytogenetic rearrangements. [11,12] Yet, both FISH and MLPA approaches are targeted and, as such, only allow analysis of specific chromosome regions correlated to a suspected clinical syndrome, which can be recognized by characteristic clinical features. The cytogenetic origin of a number of well-known syndromes has been revealed by extensive cytogenetic examination of large cohorts of individuals with similar clinical characteristics, for example velo-cardio-facial syndrome, caused by a interstitial deletion of 22q11.2, Williams-Beuren (7q11.23) and Miller-Dieker syndrome (17p13.3). [13-19] Most often the submicroscopic aberration was revealed after association of balanced translocations with the clinical phenotype. [20,21]

However, in the majority of patients with developmental delay a normal karyotype is seen and no specific submicroscopic syndrome diagnosis can be made. The gap between the demand

for whole genome analysis on one side and the possibility to detect small aberrations on the other side has been closed by the introduction of microarray techniques, also referred to as molecular karyotyping. [22-24] This whole genome technology is able to detect chromosomal aberrations at a resolution beyond the detection level of conventional karyotyping and has therefore been one of the most significant changes in the diagnostic process of individuals with intellectual disability and/or congenital anomalies. [25-27] An overview of current cytogenetic and molecular techniques used in clinical cytogenetics is given in Table 1.1.

Using new molecular karyotyping techniques, many new microdeletion and microduplication syndromes have been identified and the list is still growing. [28-31] Whereas in the pre-microarray era a clinical syndrome diagnosis was made first, followed by a confirmation on chromosome level, the expansion of high resolution whole genome techniques led to a shift from the original 'phenotype first' approach, to a 'genotype-first' approach, a development also labeled as 'reverse phenotypics'. [32] High resolution genotyping rapidly found its way as a first screening test in the daily diagnostic process of individuals with intellectual disability and/or congenital abnormalities.

In addition, these techniques are of great value in improving genotype-phenotype studies of patients with microscopically visible chromosomal imbalances by precisely determining the genomic region affected. The exact determination of breakpoints needed for genotype-phenotype studies used to be very time-consuming and only feasible for rather common cytogenetic syndromes. Examples are the determination of the Wolf-Hirschhorn syndrome critical region on chromosome 4p and the cat-cry region on chromosome 5p in Cri du Chat syndrome. [33,34] Nowadays, size and localization of the chromosomal aneuploidies can be determined with very high accuracy by whole genome oligonucleotide- and/or SNP-based arrays [35-40], reaching a resolution as low as 1 kb for detecting copy number variations (CNV).

## Deconstructing chromosomal syndromes

With the use of new molecular techniques, various chromosomal syndromes have been analyzed in detail. Whereas in some a single gene appeared to be responsible for most of the phenotypic features, for other syndromes an increasing number of critical regions for specific clinical features can be determined. In this section the detection of critical regions and some candidate genes in a number of microscopically visible chromosome disorders are described. Subsequently, some examples are given of submicroscopic aberrations in which single genes appear to play a major role in the phenotypes of patients.

**Table 1.1** Overview of techniques used in clinical cytogenetics

| Technique | Resolution; Minimal deletion sizes to be detected | Detectable level of mosaicism | Detection of balanced aberrations | Minimal turn-around time | Additional requirements | Relative estimated costs |
|---|---|---|---|---|---|---|
| Conventional karyotyping | ≥ 5 - 10 Mb | Depending on number of cells examined; ≥ 10% | Possible | 3 –10 days | • Experienced personnel for correct interpretation | Low |
| FISH | 100 kb | Depending on probe quality; ≥ 10% | Possible | 1 – 2 days | • Clinical indication of possible loci responsible for the phenotype<br>• Labour intensive | High Depending on number of annual investigations |
| Multicolour FISH/SKY | 2 – 3 Mb | ≥ 10% | Possible | 1 –7 days | • Clinical indication of suspected loci responsible for the phenotype | High |
| Comparative genomic hybridisation | ≥ 3 – 10 Mb | ≥ 50% | Not possible | 5 –7 days | • Experienced personnel<br>• Labour intensive | High |
| MLPA | ~ 0.1 kb | ≥ 40% | Not possible | 1 –2 days | • Clinical indication of possible loci responsible for the phenotype | Low; depending on number of annual investigations |

| Technique | Resolution; Minimal deletion sizes to be detected | Detectable level of mosaicism | Detection of balanced aberrations | Minimal turn-around time | Additional requirements | Relative estimated costs |
|---|---|---|---|---|---|---|
| BAC array | Depending on number of clones; 100 kb – 1 Mb | Depending on size & sort of aberration and array coverage; ≥ 30% | Not possible | 3 days | • Sophisticated equipment<br>• Standardized storage system<br>• Thorough statistical support<br>• Experienced personnel | High |
| Oligonucleotide array | Depending on number of clones; 1 – 250 kb | ≥ 10% for whole chromosome, ≥ 20-30% for segmental aneuploidies | Not possible | 3 days | • Sophisticated equipment<br>• Standardized storage system<br>• Experienced personnel for correct interpretation | High |
| SNP array | Depending on number of clones; 10 – 250 kb | ≥ 10% for whole chromosome, ≥ 15-25% for segmental aneuploidies | Not possible | 3 days | • Sophisticated equipment<br>• Standardized storage system<br>• Experienced personnel for correct interpretation | High |
| Combined platform | Depending on number of clones; 1 – 250 kb | ≥ 10% for whole chromosome, ≥ 15-25% for segmental aneuploidies | | 3 days | • Sophisticated equipment<br>• Standardized storage system<br>• Experienced personnel for correct interpretation | High |

## Cri du Chat syndrome (5p-)

Cri du Chat syndrome (CDC, OMIM 123450) was first described by Lejeune and co-workers in 1963. [41] The syndrome is caused by a partial deletion of the short arm of chromosome 5 and is characterized by a high-pitched cat-like cry, microcephaly, facial dysmorphology and intellectual disability. [42] Chromosome analysis showed different deletion sizes, but no clear association between deletion size and the clinical features could be demonstrated. [43] In 1978, Niebuhr made an attempt to locate the genetic segment responsible for the clinical features of Cri du Chat syndrome by investigating 35 individuals with a 5p- karyotype. [33] He concluded that the typical features of this syndrome were probably caused by a deletion of the midportion of the 5p15 segment, more specifically 5p15.2. This region is shown in the schematic overview in Figure 1.1. These findings have subsequently been confirmed by other groups. [44-47]

In 2005, Zhang and co-authors applied the new array CGH technique to analyze genomic DNA of 94 patients with known deletions of 5p. [48] As a detailed clinical description of all patients was available, the authors were able to define three critical regions for the cry, speech delay, and facial dysmorphology on 5p15.31, 5p15.32-15.33 and 5p15.2-15.31, respectively.

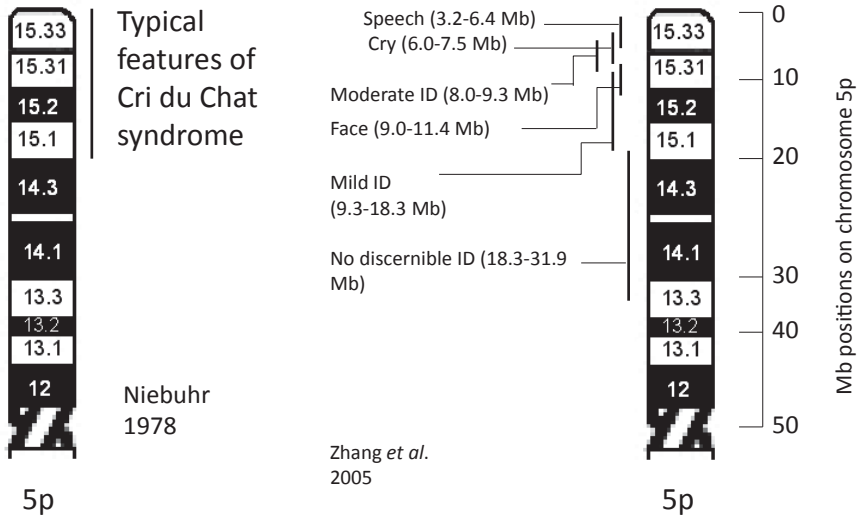


**Figure 1.1** Schematic overview of the clinical features of Cri du Chat syndrome and the associated critical regions on chromosome 5p. Array CGH was used in the study shown on the right, resulting in a significant refinement of the critical regions. ID =Intellectual Disability.

Moreover, they concluded that there were three adjacent regions on chromosome 5p that have differential effects on the level of intellectual disability (ID) if deleted. A distal 1.2 Mb deletion in 5p15.31 produces moderate ID, whereas isolated deletions of more proximal located regions result in mild or no discernible ID.

In Figure 1.1 an overview of critical regions associated with the different clinical features is provided.

## Wolf-Hirschhorn syndrome (4p-)

In 1965, groups led by Wolf and Hirschhorn each described a patient with a deletion of the short arm of chromosome 4p presenting with growth delay, intellectual disability, and congenital anomalies suggestive of a midline fusion defect. [49,50] Numerous case-reports on similar patients followed. One of the first studies in which the investigators tried to localize the segment of chromosome 4p associated with the clinical features of Wolf-Hirschhorn syndrome (WHS, OMIM 194190) was published in 1981. [51] Giemsa-banding (GTG) was performed in 13 patients. The authors concluded that the critical region involved in WHS is within 4p16, the most distal band of the p-arm (see Figure 1.2). However, not in all patients displaying the clinical features of WHS a terminal deletion could be detected by conventional karyotyping. The contribution of new molecular cytogenetic techniques, such as FISH, enabled the diagnosis of WHS in patients with submicroscopic interstitial or terminal deletions or subtle unbalanced translocations. [52,53]

A preliminary phenotypic map of chromosome 4p16 was put forward in 1995. A systematic genotype-phenotype analysis was performed in 11 patients with chromosome 4p deletions and/or rearrangements. [54] It was suggested that specific regions within 4p16 correlated with different clinical features.

In 1997 the WHS critical region (WHSCR) was refined to 165 kb by using FISH with a series of landmark cosmids in a collection of WHS patient-derived cell lines, see Figure 1.2. [34] The WHSCR is a gene-rich region and contains, among others, the *FGFR3* gene which is mutated in achondroplasia and other skeletal dysplasias.

A gene designated as Wolf Hirschhorn Syndrome Candidate 1 (*WHSC1*) was described in 1998. [55] This 25 exon gene was found to be expressed ubiquitously in early development and to undergo complex alternative splicing and differential polyadenylation. It encodes a 136 kD protein containing 4 domains also present in other developmental proteins. It is expressed

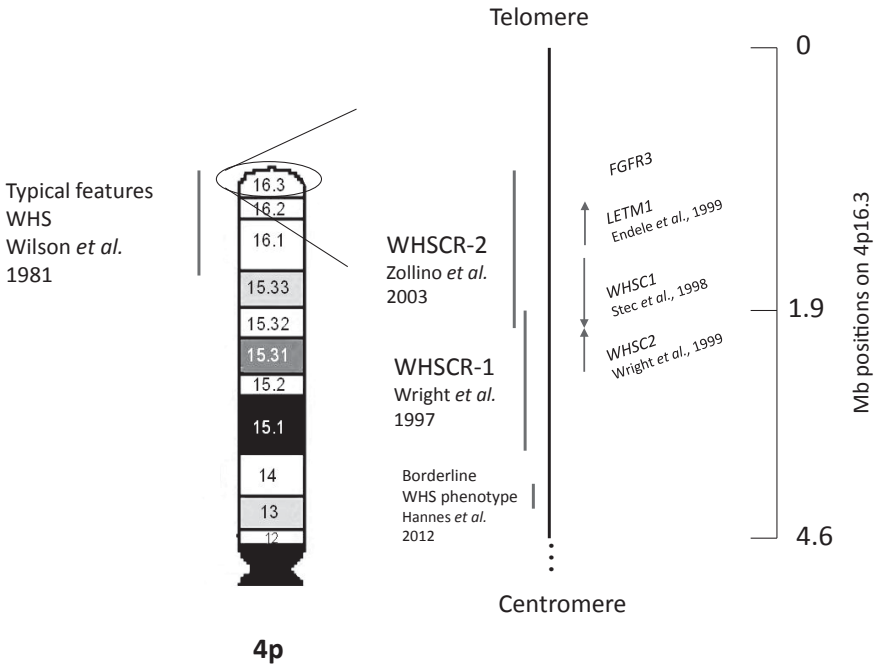


**Figure 1.2** Schematic overview of the deconstruction of the Wolf-Hirschhorn syndrome in critical regions and candidate genes. WHS = Wolf-Hirschhorn syndrome; WHSCR-1 = WHS critical region 1; WHSCR-2 = WHS critical region 2; *FGFR3* = Fibroblast growth factor receptor 3 ; *LETM1* = Leucinezipper/ef-hand-containing transmembrane protein 1; *WHSC1* = WHS candidate gene 1; *WHSC2* = WHS candidate gene 2. Mb = Megabase.

preferentially in rapidly growing embryonic tissues, in a pattern corresponding to the affected organs in WHS patients. The nature of the protein motifs, the expression pattern, and its mapping to the critical region led the authors to propose *WHSC1* as a good candidate gene for WHS. A second candidate gene (*WHSC2*) was identified one year later, as well as *LETM1*, which may contribute to the neuromuscular features of WHS patients. [56,57] The location of all candidate genes is depicted in Figure 1.2.

In 2000 an Italian group reported the cytogenetic, molecular, and clinical findings in 16 WHS patients. [58] Submicroscopic deletions ranging from 2.8 to 4.4 Mb were detected in four patients. In one patient, no molecular deletion could be detected within the WHSCR. The precise definition of the cytogenetic defect permitted an analysis of genotype/phenotype correlations in WHS, leading to the proposal of a set of minimal diagnostic criteria. Deletions of less than 3.5 Mb resulted in a mild phenotype in which major malformations were absent.

The authors proposed a 'minimal' WHS phenotype in which the clinical manifestations are restricted to the typical facial appearance, mild intellectual disability, growth retardation, and congenital hypotonia.

In 2003, the same group reported their findings in eight patients carrying a 4p16.3 microdeletion. [59] The WHSCR was fully preserved in one patient with a 1.9 Mb deletion, in spite of a typical WHS phenotype. Therefore, the authors proposed a second critical region, WHSCR2, a 300 kb interval located distally from the known WHSCR1 (Figure 1.2). Furthermore, for the purpose of genetic counseling, they recommended to divide the WHS phenotype into two distinct clinical entities, i.e., a 'classical' and a 'mild' form, which are usually caused by cytogenetically visible and submicroscopic deletions, respectively. Another patient with a 1.9 Mb subtelomeric deletion was described in 2005, which supports the proposed WHSCR2. [60]

A Belgian group reported six additional patients with an atypical 4p16.3 deletion, of whom five patients showed a (very) mild form of WHS and one patient had no clinical signs of WHS. [61] By means of a contiguous 4pter BAC array, the sizes and breakpoints were physically mapped and 4 terminal deletions (range 0.4-3.81 Mb) and 2 interstitial deletions (1.55 and 1.7 Mb) were revealed. This study enabled further refinement of the phenotypic map of this region, suggesting hemizygosity of *WHSC1* to cause the typical WHS facial appearance.

Recently a 432 kb deletion located 600 kb proximal to both WHSCR1 and WHSCR2 has been identified in a patient and his mother with a WHS facial phenotype (Figure 1.2). [62] Sanger sequencing of *WHSC1* and *WHSC2* did not reveal any mutations. The authors hypothesize that either this locus harbors regulatory sequences which affect the expression of genes in WHSCR1 and WHSCR2 or, alternatively that this locus is another genetic locus co-segregating with the WHS phenotype. They concluded that the microdeletion leads to a borderline WHS phenotype and propose that this locus predisposes to WHS.

In summary, although molecular analysis allows a more detailed view of the WHS critical regions, the exact contribution of each of the proposed critical regions to the WHS phenotype still remains to be determined.

## 18q deletion syndrome

The 18q deletion syndrome (OMIM 601808) was described first in 1964 by De Grouchy et al. [63] Most 18q cases are associated with terminal deletions and the phenotype of this syndrome is

mainly characterized by neonatal hypotonia, intellectual disability, facial dysmorhpisms, ear canal anomalies and foot deformities. A first preliminary phenotypic map based on seven patients with deletions of 18q21.3 or 18q22.2 to 18qter was published in 1993. [64] In Figure 1.3 an overview of clinical features and associated chromosome regions is provided.

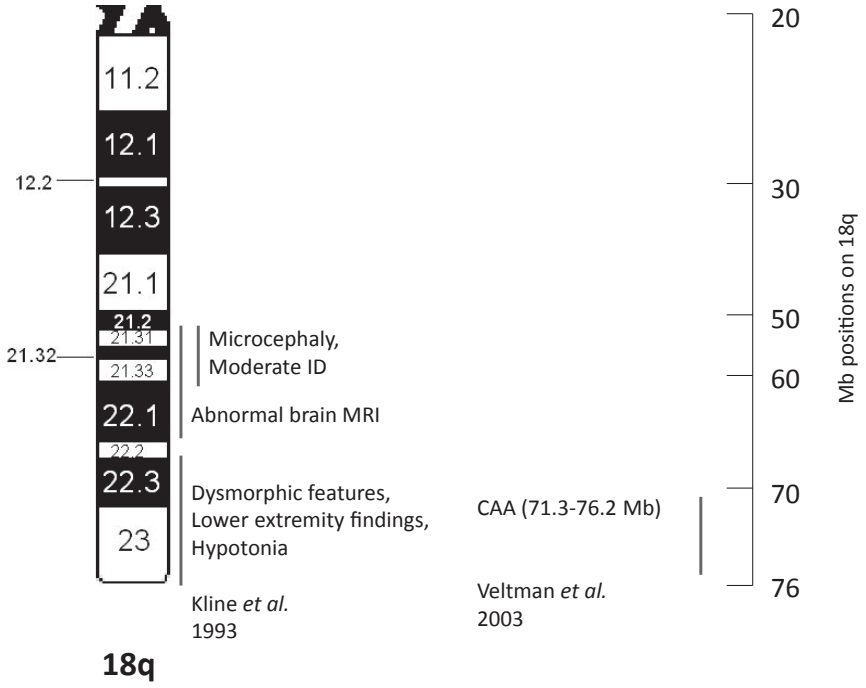


**Figure 1.3** Overview of the long arm of chromosome 18 and the critical regions defined for distinctive clinical features. ID = Intellectual Disability; CAA = congenital aural atresia.

A substantial percentage of 18q- patients has congenital aural atresia (CAA), leading to hearing loss. [65] By applying a 670 kb resolution chromosome 18-specific BAC array to analyse genomic DNA of 20 patients with CAA, a critical region for CAA was mapped on 18q22.3-18q23. [66] Of these, 18 patients had a microscopically visible 18q deletion. In 2 patients, a submicroscopic 18q deletion was detected which allowed the mapping of CAA to a region of 5 Mb located in 18q22.3-18q23 (Figure 1.3).

## 1p36 deletions

One of the relatively new microdeletion syndromes, as this aberration has only been known since 1997, is the 1p36 deletion syndrome. [67] With an estimated prevalence of 1

in 5,000 live births, monosomy of 1p36 (OMIM 607872) is the most common terminal deletion syndrome. [68] Because of the variability in deletion size, parental origin and clinical presentation, it has been proposed that monosomy 1p36 is a contiguous gene syndrome in which haploinsufficiency of functionally unrelated genes leads to the phenotypic features. [69]

The first physical map of 1p36 deletions was published in 2003. [70] First, DNA samples of 61 patients were screened with 25 microsatellite markers for the most distal part of 1p36. Then, a contig of 99 overlapping large-insert clones of this 10.5 Mb region was used to further refine the deletion size. Furthermore, clinical phenotypes of 30 patients were carefully defined. The authors proposed critical regions for hypotonia (2.2 Mb region from the telomere), a large fontanel (2.2 Mb), hearing loss (2.5 Mb), cardiomyopathy (3.1 Mb), hypothyroidism (4.1 Mb) and clefting (4.1 Mb). Because the terminal region of 1p36 is gene rich, no candidate genes could be determined.

In the same year, this group published their data using a dedicated 1p36 array CGH. [71] This array was designed by using the previously assembled contig, consisting of 97 clones from 1p36, supplemented by clones for the subtelomeric regions of all chromosomes and clones for both sex-chromosomes. Genomic DNA of twenty-five patients with well-defined 1p36 deletions was studied and the array results agreed with the previously determined deletion sizes and breakpoint locations as detected by FISH and microsatellite analyses.

Recently, a tiling resolution BAC array covering 99.5% of the euchromatic parts of chromosome 1 has been applied to study six patients with a 1p36 deletion phenotype. In all patients a 1p36 deletion was confirmed, with sizes ranging from 2 to 10 Mb. Remarkably, in two clinically similar patients two non-overlapping deletions were detected. Therefore, the authors concluded that the 1p36 phenotype is a consequence of distinct and non-overlapping deletions having a positional effect rather than being a true contiguous gene deletion syndrome. [72] Battaglia and collagues evaluated the clinical features in a large series of 60 patients with the 1p36 deletion syndrome in order to thoroughly delineate the natural history with the purpose of developing complete and accurate information that can be used for answering families' questions in the clinical setting. [73] The 1p36 deletions were detected by regular karyotyping, FISH of the subtelomeric region or array comparative hybridization, yet the authors did not mention specific critical regions for the various clinical features described.

## Cytogenetic microdeletion syndromes and the impact of single genes

In a number of (micro)deletion syndromes, the molecular determination of breakpoints together with a comparison of clinical features has resulted in such small critical regions that single genes appear to be responsible for the (majority of) phenotypic features.

An example is Smith-Magenis Syndrome (SMS, OMIM 182290), characterized by behavioural problems, speech delay, psychomotor and growth retardation and distinct craniofacial features. [74] About 75% of the SMS patients have a common deletion spanning 3.5 Mb in the 17p11.2 region, although deletion sizes vary from 1.5 Mb to 9 Mb. [75,76] Successively a number of patients who fulfill the criteria for SMS but without the 17p11.2 deletion were analyzed for mutations of *RAI1*, located within the central portion of the critical region for SMS, using PCR and sequencing strategies. [77-79] This resulted in the identification of nine patients having *RAI1* mutations and to the conclusion that haploinsuffiency of this gene is associated with the craniofacial, behavioral and neurological symptoms of SMS.

The 22q13.3 deletion or Phelan-McDermid syndrome (OMIM 606232) is characterized by neonatal hypotonia, severe expressive language delay in combination with mild intellectual disability. [80,81] Included in the critical region of this syndrome is *SHANK3*, which is preferentially expressed in the cerebral cortex and the cerebellum. DNA analysis of *SHANK3* in a patient carrying a de novo balanced translocation between chromosomes 12 and 22, t(12;22)(q24.1;q13.3), revealed a disruption within exon 21. [82] Since the patient displayed all 22q13.3 deletion features, the authors proposed that *SHANK3* haploinsufficiency is the cause of the 22q13 deletion syndrome. This finding was supported by another group who tested 45 patients with variable sizes of 22q13 deletions, thereby confirming a deletion for the *SHANK3* gene in all patients. [83] An array CGH study for molecular characterization of nine patients with 22q13 aberrations identified deletion sizes ranging from 3.3 to 8.4 Mb. [84] The authors did not observe a relation between clinical features and deletion size, thereby supporting the idea that a gene in the 3.3 Mb minimal deleted region, notably *SHANK3*, may be the major candidate gene in the 22q13 deletion syndrome. Another group using array CGH reported their findings in two unrelated 22q13.3 deletion patients [85], which were consistent with the concept of *SHANK3* being the best candidate gene for the neurological deficits in the 22q13.3 syndrome [86], although patients with the same kind of *SHANK3* disruption can exhibit different degrees of severity in their phenotype.

Another terminal deletion syndrome is the 9q34 subtelomeric deletion syndrome. This syndrome is characterized by severe intellectual disability, hypotonia, microcephaly

and a typical face with midface depression, hypertelorism, everted lower lip, cupid bow configuration of the upper lip, and a prominent chin. The minimum critical region involved is ~1.2 Mb in size and encompasses at least 14 genes. [87] In a mentally retarded patient with a typical 9qter deletion phenotype, a balanced translocation t(X;9)(p11.23;q34.3) was detected. [88] Extensive analysis of the breakpoints revealed a disruption of *EHMT1*, indicating that haploinsuffiency of this gene may be responsible for the 9q subtelomeric deletion syndrome. Subsequently, sequence analysis of *EHMT1* in a series of patients with clinical phenotypes suggestive of a 9qter deletion but with intact telomere region according to FISH and MLPA, was performed. This resulted in a *de novo* nonsense mutation in one such patient and a frameshift in another, establishing that *EHMT1* haploinsufficiency is indeed the cause of the 9qter deletion phenotype. [89] Numerous patients with an *EHMT1* mutation have been identified since then.

Recently, Koolen and colleagues showed that haploinsufficiency of *KANSL1* is sufficient to cause the 17q21.31 microdeletion syndrome, a multisystem disorder which is characterized by intellectual disability, severe neonatal hypotonia an amiable personality and distinctive facial features. [90] They were able to delineate the critical region from five to two known genes by using the Affymetrix Cytogenetics Whole Genome 2.7M array, which detected a very small and atypical deletion in each of two patients with a classical 17q21.31 phenotype. The overlapping region encompassed only parts of *MAPT* and *KANSL1*, in which Sanger sequence analysis revealed loss of function mutations in *KANSL1* in two other individuals with a comparable phenotype yet without a 17q21.31 deletion.

Other examples of cytogenetic syndromes of which the (majority of) clinical features appear to be caused by mutations in single genes are Rubinstein-Taybi Syndrome (RSTS, OMIM 180849), Sotos syndrome (OMIM 117550) and DiGeorge/VCFS Syndrome (DGS, OMIM 188400). [91-95] Furthermore, it has been described that atypical deletions may be associated with variant phenotypes. [96,97]

These examples illustrate how the boundary between cytogenetic deletion syndromes and single gene conditions is becoming more and more indistinct. Ultimately, we should be able to assess the phenotype contribution of each gene within known microdeletion / microduplication syndromes.

An overview of the above-mentioned syndromes and the possible genes responsible for most phenotypic features is given in Table 1.2.

**Table 1.2** Examples of cytogenetic microdeletion syndromes in which single genes appear to be responsible for the (majority of) clinical features

| Syndrome | Chromosome location | Gene responsible |
|---|---|---|
| Sotos Syndrome | 5q35 | *NSD1* |
| Kleefstra syndrome | 9q34 | *EHMT1* |
| Rubinstein-Taybi Syndrome | 16p13.3 | *CREBBP, EP300* |
| Smith Magenis Syndrome | 17p11.2 | *RAI1* |
| Koolen-De Vries syndrome | 17q21.31 | *KANSL1* |
| DiGeorge/VCFS Syndrome | 22q11.2 | *TBX1* |
| Phelan-McDermid syndrome | 22q13.3 | *SHANK3* |

In summary, the examples above show three different forms of associations between chromosomal disorders and a phenotype. In a number of syndromes the phenotype is primarily caused by disruption of a single gene, like Rubinstein-Taybi syndrome. In contrast, Cri du Chat syndrome is a contiguous gene deletion syndrome as the different clinical features seem to be caused by deletions of non-overlapping critical regions. A third mechanism is seen in Wolf-Hirschhorn syndrome, where the identification of two critical regions for the complete phenotype, shows that disturbance of the integrity of a chromosomal region can lead to a recognizable phenotype.

## Mapping of malformations by chromosomes; Mendelian cytogenetics

The first successful mapping of a Mendelian disorder by chromosome rearrangements was that of the Duchenne muscular dystrophy, located at Xp21. [98] Since then, chromosome aberrations which delete, truncate, or otherwise rearrange and alter specific genes have not only helped in the mapping of other disease loci, but have turned out to be key elements for the rapid isolation of disease genes by positional cloning strategies. [99,100] Tommerup documented that the frequency of associated chromosome rearrangements in Mendelian disorders may be rare, however not exceptional. [101]

Using a mathematical model, chromosome maps for specific malformation patterns based on the catalogue of unbalanced chromosome disorders and associated congenital malformations collected in the Zurich Cytogenetic Database were created. [102,103] The chromosomal deletion

map was assembled through the analysis of 1,753 patients with a single, non-mosaic contiguous autosomal deletion and the presence of common major malformations. This resulted in 284 positive associations between specific malformations and deleted bands, distributed among 137 malformation-associated chromosome regions (MACRs). In a second article, a chromosomal duplication map was described. [103] Here, a total number of 143 MACRs were identified, of which 21 were highly significant.

Obviously, such maps should always be interpreted with care. Although the number of cases available for analysis was high, the accuracy of breakpoints is not known since the cytogenetic analyses were mostly performed with standard karyotyping. Nonetheless, this type of analyses can point to those chromosome regions where the search for loci involved in congenital malformations is most likely to be successful. This has been abundantly proven for holoprosencephaly, where at least four genes have been found based on chromosomal mapping of critical regions. [104-109]

As more and more submicroscopic deletions and duplications are mapped, further candidate genes for specific malformations are being revealed. For instance, a study in 100 patients with intellectual disability and malformations detected a small duplication in 5q35.1 in a patient with lobar holoprosencephaly. [25] This region contains seven known genes of which *FBXW11* is a likely candidate gene for holoprosencephaly. [110]

## Storage of genomic and clinical data

Cytogenetic and clinical information concerning specific chromosome disorders are continuously published in the (inter)national medical literature. Thus, systematic collection and archiving are essential.

Many of these reports have been collected in the 'Catalogue of Unbalanced Chromosome Aberrations in Man', containing around 2,000 descriptions of patients with a rare chromosome aberration. [10] This catalogue provides an unprecedented resource for genotype-phenotype studies in cytogenetically visible chromosome anomalies. In order to perform searches directed towards specific chromosome aberrations and/or clinical features, a computerized version is commercially available as the Zurich Cytogenetic Database, which contains cytogenetic and clinical information on more than 7,200 cases from the medical literature and references to the original papers. [111]

## What about the phenotype?

Abnormal phenotypes have played significant roles in the discovery of critical chromosome regions and gene function, but organized collection of phenotype data has been overshadowed by developments in genetic technology. As high resolution genotyping is currently part of the standard genomic testing in individuals with intellectual disability and/or congenital abnormalities in developed countries, there is a need for equally high accuracy of phenotyping to fully benefit from the advantages of these new techniques.

The phenotype, defined as the appearance (physical, biochemical and physiological) of an individual which results from the interaction of the environment and the genotype, is usually presented in scientific articles by a clinical description, sometimes accompanied by clinical photographs. Any description of clinical features of a patient is inherently subjective. It varies between independent physicians and any emphasis on specific features may reflect the background specialty of the observer.

## Description of phenotypes

To overcome the bias of subjectivity, proposals have been made to standardize the phenotypic description by a systematic collection of clinical information. [112-114]

A detailed proposal for the organization and standardization of clinical descriptions of human malformations has been made by Biesecker. [115] The author felt that, in contrast to the enormous improvements in molecular biology, the processes and approaches of the clinical component of molecular dysmorphology have not changed substantially. He argued that the current way of collecting phenotypic information holds several weaknesses. The quality and completeness of clinical descriptions published in the medical literature depend on the authors and editors involved. Another threat is confusion in understanding the terms used by the authors, due to the existence of synonyms, various definitions for one word, and sometimes overlapping of two different terms. The author pointed out a number of criteria for an ideal standardized clinical genetics nomenclature.

An international working group was subsequently formed to develop standardized definitions and terms to describe the physical variations used in human phenotypic analyses. This project, which came to be known as the *Elements of Morphology*, resulted in six articles proposing consensus definitions for almost 400 phenotypic variations of the head and face; periorbital region; ear, nose, and philtrum; mouth and lips; and hands and feet. [116-121] Every variation was

accompanied by a representative figure depicting the feature. As a result, several human and medical genetic journals implemented the inclusion of the use of the *Elements* terminology into manuscripts submitted to their journals.

Currently the group is working on four more articles on proposed terminology for the trunk, genital region, skin, and remainder of the limb. [122]

Standardization of phenotype descriptions will be crucial for a 'Human Phenome Project', in which comprehensive databases are created for such systematically collected phenotypic information. [112] The authors argued that phenotypic information should be collected on different levels: molecules, cells, tissues and whole organisms.

## Visualization of phenotype

In a number of cytogenetic syndromes, such as WHS or 1p36 deletion syndrome, the clinical diagnosis is primarily based on characteristic facial features. Clinical geneticists are trained in recognizing specific patterns in different syndromes and can do this relatively well. [123] Multiple efforts have been made to implement objective, quantitative criteria and analytical techniques for craniofacial assessments. [124,125] In previous decades, anthropometry, photogrammetry and cephalometry have been applied as diagnostic methods. [126-129]

More recently, computer programs have been designed to analyze and identify faces of patients with certain syndromes on the basis of specific craniofacial features. In one study, standardized photographs of 55 patients with different syndromes were analyzed in a mathematical way by comparing feature vectors at 32 facial nodes. [130] Over 75% of the patients were correctly classified by the computer, whereas clinicians who were shown the same pictures achieved a recognition rate of 62%.

In 2005, a large study on computer-based three-dimensional (3D) imaging of the face of 696 individuals was published. [131] This study demonstrated the potential contributions of dense surface models (DSM) in clinical training, making clinical diagnoses and objective comparisons. Such mathematical pattern recognition might improve phenotype-genotype analyses, particularly in patients with rare or atypical chromosome aberrations.

A first application of 3D face surface models in genotype-phenotype studies was demonstrated in Williams-Beuren syndrome (WBS, OMIM 194050), involving a 7q11.23 deletion. [132] As the typical deletion size in WBS is 1.5 Mb and contains 28 genes, a clear genotype-phenotype

correlation for craniofacial features could not be made so far. In this study, a patient with a small, atypical deletion was identified and 3D surface images of this patient's face were compared with those of WBS-individuals and controls. The patient was classified as borderline WBS with mildly dysmorphic features. Chromosome analysis revealed a heterozygous deletion at 7q11.23 of ~1 Mb, resulting in reduced expression of *GTF2IRD1*. In mice, homozygous loss of *Gtf2ird1* results in craniofacial abnormalities reminiscent of those seen in WBS, together with growth retardation. These observations suggest that *GTF2IRD1* plays a role in mammalian craniofacial and cognitive development. The authors suggested that cumulative dosage of TFII-I family genes explains the main phenotypes of WBS. *Gtf2ird1*-null mice and classic WBS patients have two functional copies (in trans and cis, respectively), whereas the atypical patient had three functional genes of the *GTF2IRD1*/*GTF2I* cluster and showed a milder WBS phenotype.

Recently, DSM and pattern recognition techniques were used to compare the facial phenotype of WHS in individuals with a small terminal deletion (breakpoint within 4p16.3) compared to those with a large deletion (breakpoint more proximal than 4p16.3). [133] By using these sensitive and accurate visualization and quantitative tools it was demonstrated that small terminal deletions are associated with milder facial dysmorphology than large deletions. Further, fine-grained facial analysis of several individuals with an atypical genotype and/or phenotype suggested that multiple genes contiguously contribute to the characteristic Wolf–Hirschhorn syndrome facial phenotype, as suggested previously. [61]

## Aims and outline of the thesis

The main aim of this thesis was to provide genotype-phenotype relationships for rare chromosomal abnormalities. Such genotype-phenotype relationships provide valuable information for clinicians and molecular cytogeneticists working in the fields of clinical genetics and prenatal diagnosis. They may further add to disease gene discovery.

As there is a need for sophisticated phenotyping and data collection, we started by setting up a web-based genotype-phenotype database for rare chromosomal aberrations which is accessible for everyone working in this field, in order to increase the level of knowledge among physicians and their patients (***Chapter 2***).

The detection of submicroscopic chromosomal abnormalities (CNVs) by arrays is well established in the evaluation of children with significant learning disability or major

malformations. Whether arrays would provide relevant information in patients with karyotypically balanced rearrangements, or in patients with prenatally detected structural abnormalities on ultrasound is largely unknown. These situations are especially challenging.

In ***Chapter 3***, we therefore evaluated the use of high resolution whole genome arrays in the diagnostic setting of these two groups of patients, where either the genotype or the phenotype is incomplete. We studied submicroscopic imbalances in individuals with congenital abnormalities or developmental delay and a *de novo* apparently balanced translocation or inversion and applied a predictive clinical scoring system in ***Chapter 3.1***. In ***Chapter 3.2***, a study on the evaluation of the clinical and laboratory aspects of non-targeted whole genome array analysis for prenatal diagnosis of foetuses with structural anomalies is described.

Detailed genotype-phenotype analysis of small chromosomal imbalances may point to specific genes for the phenotype or a component of a complex phenotype. By comparing multiple small CNVs a chromosomal phenotype map can be generated that allows the mapping of such genes. We decided to study a large cohort of patients with overlapping 18q deletions in order to generate an upgrade to the existing phenotypic map, and to study neuropsychiatric aspects and the occurrence of congenital heart defects. Results are reported in ***Chapters 4.1***, ***4.2*** and ***4.3***, respectively.

After generating this 18q deletion map, we studied specific subphenotypes linked to single genes on 18q. A chromosome translocation leading to disruption of *TCF4* in a female patient with mild to moderate intellectual disability and minor facial anomalies was studied and compared to previously reported patients with Pitt Hopkins syndrome in ***Chapter 5.1***.

In addition, we wanted to determine whether the features of the distal 18q deletion syndrome are caused by disruption of one single gene, or should be considered as a true contiguous gene deletion syndrome (***Chapter 5.2***).

Finally, the implications of this work and future directions are discussed in ***Chapter 6***.

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chapter 2

# European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA); an online database for rare chromosome abnormalities

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## ABSTRACT

During recent years a considerable improvement in diagnostic techniques has enabled cytogeneticists to find more and smaller chromosomal aberrations. However, accurate clinical knowledge about rare chromosome disorders is frequently lacking, mostly due to a significant decline in publishable cases. On the other hand, there is an increasing demand from parents and physicians for reliable information. In order to improve the quality and the quantity of data available, we designed a new database named the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA) at http://www.ecaruca.net. This Internet-database contains cytogenetic and clinical data of patients with rare chromosome abnormalities, including microscopically visible aberrations, as well as microdeletions and -duplications. Cases with certain breakpoints collected in the Zurich Cytogenetic Database were transferred to ECARUCA. The advantages of ECARUCA compared to existing sources are that ECARUCA is interactive, dynamic and has long-term possibilities to store cytogenetic, molecular and clinical data. Professionals can login to submit new cases and perform searches in the database through the Internet. Currently the database contains 1500 unique chromosomal aberrations from almost 4000 patients. A frequent submission of new data ensures the up-to-date quality of the collection. Individual parent accounts allow parents to inform the ECARUCA team about the follow-up of their child. The ECARUCA database provides health care workers with accurate information on clinical aspects of rare chromosome disorders. Additionally, detailed correlations between chromosome aberrations and their phenotypes are of invaluable help in localising genes for mental retardation and congenital anomalies.

## INTRODUCTION

Up to 0.6% of the general population have an unbalanced chromosome aberration. [1] Depending on the diagnostic methods used and the population studied, microscopically visible chromosomal aberrations occur in 4–28% of mentally retarded patients, making chromosomal abnormalities a major cause of mental retardation. [2,3] Relatively common conditions like Turner syndrome and Down syndrome are clinically well known and recognisable, in contrast to the very limited knowledge on many rare chromosome abnormalities.

These rare chromosome disorders have a total incidence of at least 0.07% [4] and with an annual birth rate of slightly more than 7 million in Europe [5], it can be estimated that currently 3000–5000 children with a rare chromosome aberration are born each year on this continent only.

Moreover, the number of more or less unique chromosomal aberrations that are identified is rapidly increasing due to the development of new molecular and cytogenetic techniques such as multiplex ligation-dependent probe amplification (MPLA), multiplex amplifiable probe hybridisation (MAPH) and array-based comparative genomic hybridisation (array CGH). [6-9]

Subtelomere screening by fluorescence in situ hybridisation (FISH) and genetic markers resulted in the identification of submicroscopic subtelomeric rearrangements in approximately 5% of mentally retarded patients. [10,11] More advanced techniques such as genome wide high resolution array CGH, will allow the detection of submicroscopic interstitial deletions and duplications, which consequently will considerably increase the number of detectable chromosomal aberrations.

However, while the number of chromosomally defined syndromes is rising, the clinical knowledge about individual syndromes remains limited due to the low number of patients that will actually be published in detail.

The source for clinical information concerning specific chromosomal disorders that is available for clinicians, counsellors, researchers and parents is the (inter)national medical literature. The main sources of information in scientific journals are case reports and occasionally a review discussing a chromosomal syndrome like Wolf–Hirschhorn or Cri du Chat. [12,13] Many reports have been collected in the "Catalogue of Unbalanced Chromosome Aberrations in Man" written by Schinzel. [4] This standard work is based on the literature of the last 30 years and contains around 2000 descriptions of patients with a rare chromosome aberration. In addition, the commercially available Zurich Cytogenetic Database, which contains over 7200 cases, can be used as a digital resource for information. [14]

Although all references mentioned are extremely valuable, the number of reports per individual chromosome aberration remains rather limited. This is mostly due to the declining number of published or publishable papers describing the clinical features of a single patient with a rare chromosome abnormality.

Furthermore, articles illustrating the diagnostic improvements of new cytogenetic techniques usually provide only a limited clinical description of a large number of patients. Moreover, patients with a rare chromosome aberration are mostly diagnosed and described in scientific journals at a (very) young age. Follow-up information in these cases is hard to obtain, resulting in a lack of valuable information.

In conclusion, there is a need for an accessible dynamic database to fulfil the demand for information from clinicians, scientists and parents about rare (sub) microscopic chromosome aberrations. The Internet has been chosen as the medium for establishing the interactive online European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (ECARUCA). In this article the development of the ECARUCA project and the structure of the database are described.

## MATERIALS AND METHODS

### Objectives

The objectives of ECARUCA are to improve two supplementary areas: the medical and scientific working fields of rare chromosome aberrations (see Figure 2.1).

The ECARUCA project aims to increase knowledge on clinical features in patients with rare chromosome aberrations. Within the medical field, physicians, genetic counsellors and other health care providers are explicitly calling for an improvement of availability of medical and psychosocial information. Due to the absence of detailed knowledge on the clinical features and follow-up in rare chromosome aberrations, parents may currently not receive the optimal clinical information related to the chromosomal aberration of their child, especially concerning complications that occur later in life and information about the achievement of developmental milestones.

Furthermore, contact between parents of children with a similar chromosome aberration has shown to be very valuable and should be facilitated.

**Medical issues**

- Increase of information on clinical features and complications in patients with (sub)-microscopic chromosome aberrations
- Encourage and facilitate contact between patients & parents
- Support less facilitated centres specifying chromosome aberrations

**Scientific issues**

- Compile a resource of biological material useful for mapping candidate genes for specific clinical abnormalities and identification new syndromes
- Enhance co-operation between centres

**Figure 2.1** The main objectives of the ECARUCA project.

For the reliability of the database the accuracy of the cytogenetic studies performed in each case is very important. Therefore ECARUCA has set up a program for less facilitated centres to get free support in further cytogenetic characterisation of patient samples. Through this program, the number of cases in the database will increase. Moreover, exact determination of the breakpoints enables the medical professional to search for clinical information described in similar patients, thereby informing parents more accurately.

For scientists, ECARUCA aims to be a resource of information that is useful in localizing candidate genes as well as the identification of new syndromes. As (cytogenetic) techniques improve and smaller, unique genomic aberrations are detected, the collective registration of the clinical features occurring in these patients is of utmost importance. Therefore, cooperation between different centres and the exchange of knowledge will be initiated by ECARUCA.

Integrating the medical and scientific objectives should eventually lead to the improvement of quantity and quality of knowledge on rare chromosome aberrations available to both professionals and patients.

## European network

In order to facilitate communication and cooperation between countries, physicians and scientists, a European network has been set up (Figure 2.2).


**Figure 2.2** Overview of the European network of the ECARUCA project.

First, a National Coordinator has been assigned in each participating country. The National Coordinator acts as an intermediary between the professionals of his or her country and ECARUCA. The National Coordinator represents ECARUCA during national meetings and informs colleagues on new developments within the project. Furthermore, the National Coordinator can assist and stimulate colleagues to submit new cases to the ECARUCA database.

Sometimes, language difficulties can discourage a person to take part in an international project. To lower this threshold, the National Coordinator acts as the first contact person to whom people can turn to for information. Conversely, the Coordinator can inform ECARUCA on important developments on clinical genetics within his or her country. An up-to-date list of all National Coordinators including contact information is available on the ECARUCA website.

Another important role in the European network is fulfilled by the patient organisations. Several support groups in different European countries support the ECARUCA project. Patient organisations play an important role in supplying information to family members of patients with a rare chromosomal aberration. They also offer a contact service between patients and they can provide patient data.

Furthermore, a Microdeletion Research Network composed of several research centres around Europe has been set up. Now and in the future, collaboration between centres working on rare chromosome aberrations will be of great importance. A better understanding of the underlying genetic mechanisms and clinical outcome can only be reached through collective, standardised registration of these unique patients in one database, accessible to all participants.

## Database model and confidentiality

The cytogenetic, molecular and clinical data collected by ECARUCA is stored in a relational database management system. All cases receive a unique case ID number and all data like cytogenetic, clinical and pedigree information are linked to that ID number. The database can be queried about aberrations of chromosome regions according to the ISCN 1995 nomenclature.

The design of the ECARUCA data model also allows storage of aberrations at base pair level in order to collect molecular data of patients with a submicroscopic aberration.

The database is situated on a secure server at the Department of Human Genetics in Nijmegen. A web interface, using state of the art Java technology, enables users to view and submit data to ECARUCA safely and promptly on the website.

The security and integrity of the data is not only ensured by the correct implementation of hardware and software solutions, but also at the level of the user, by only allowing account holders to have access to the data. Accounts are granted exclusively to professionals in the field of human genetics and health care, in order to ensure confidentiality and correct interpretation of (clinical) data.

Before submission of data of a new patient, parents or the legal guardian need to sign an informed consent form. This form is stored in the patient file of the submitting centre.

# RESULTS

## Database contents

Currently, the database contains around 4000 cases with almost 1500 unique aberrations.

A large number of cases have been derived from the Zurich Cytogenetic Database, established by A. Schinzel and collaborators. This database contains cytogenetic and clinical data of published cases. Excluded are the three most frequent autosomal trisomies (for chromosomes 13, 18 and 21), and X and Y chromosome aberrations unless combined with an autosomal aberration.

Before transferring cases to the ECARUCA database, all cases with uncertain breakpoints were excluded. Breakpoints were considered to be uncertain if the original publication did not provide a complete confirmation of breakpoints in the patient described. Moreover, because of the different data models of the Zurich and ECARUCA databases, all individual aberrations needed to be redefined. In the original database they are stored as text fields, while in ECARUCA they are stored as alphanumerical values, allowing conversion to base pair level. An aberration is defined by the start and end position in base pair running from pter to qter. In this way all molecular and cytogenetic data can be optimally integrated.

Currently we are working on the implementation of query possibilities and display of data on the base pair level.

Figure 2.3 shows an overview of the number of deletions and duplications per chromosome. The aberrations are distributed over the entire genome and include the following types: deletions, duplications, rings, uniparental disomies, trisomies, triploidies and tetraploidies. The majority are deletions (N = 2296), followed by duplications (N = 1773). As expected, some chromosomes contain more abnormalities (e.g. 4, 11, 13, 15, 18, 22) than others (e.g. 12, 14, 16, 19), partly due to differences in size and gene density. Moreover, a number of chromosomes are involved in more common aberrations such as the 138 cases of DiGeorge syndrome, with a deletion of 22q11.2.

## Website

The collection and distribution of information on rare chromosome aberrations takes place through the ECARUCA website (www.ecaruca.net). The website consists of two sections:

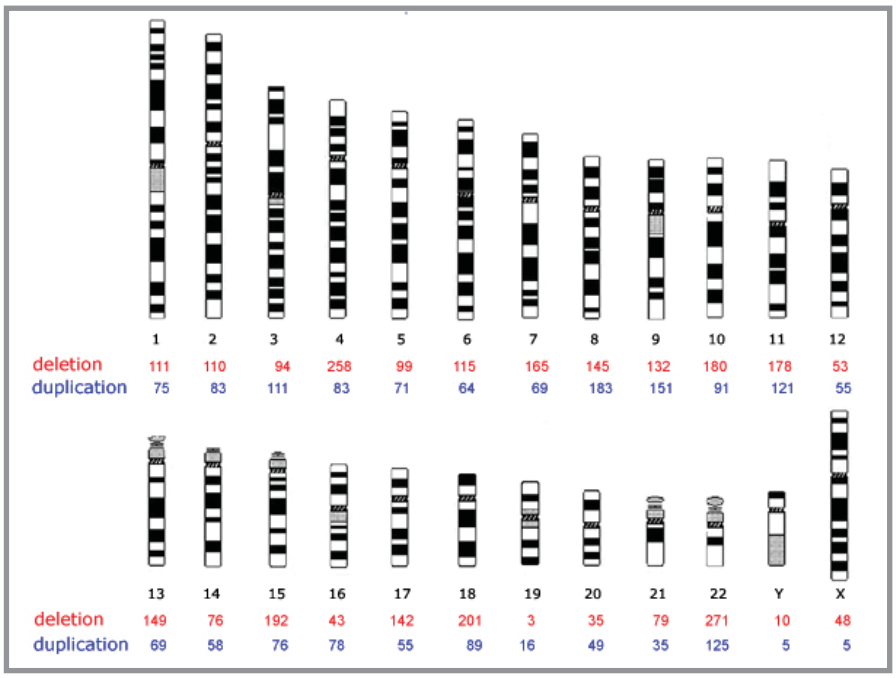


**Figure 2.3** Overview of the number of deletions and duplications per chromosome.

public pages that are freely available to each Internet user and restricted pages containing patient information that are only accessible to account holders.

Via the homepage (Figure 2.4), an Internet user can access four main areas: Submit Cases, Query Database, Cytogenetic Verification and Frequently Asked Questions. The menu on the left side of the homepage gives entry to remaining topics such as an overview of the data and a frequently changing introduction of a European patient organisation.

On a monthly basis, an interesting patient that has been submitted to the database will be published on the website. A general description of this patient is given at the public pages, whereas the restricted pages contain detailed cytogenetic and clinical information and illustrative clinical pictures. Cases considered for this topic are selected by the Clinical Database Managers (I.F. and D.A.K.) in accordance with the Project Management Board, consisting of the delegates from the centres in London, Zurich and Nijmegen. Furthermore, professionals are encouraged to bring interesting patients to the attention of ECARUCA.

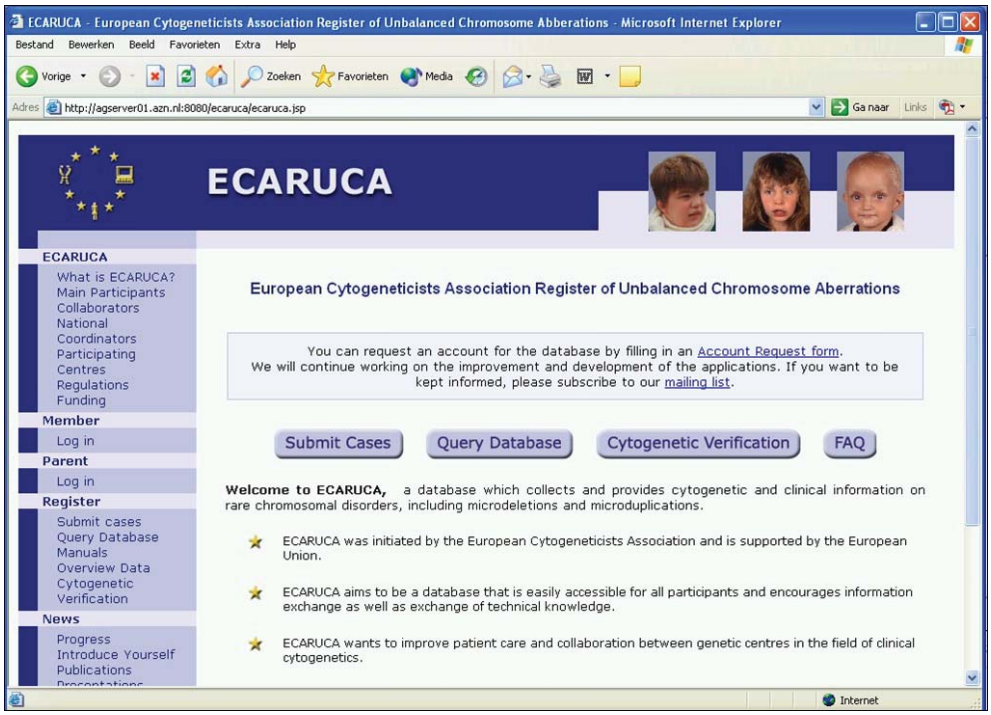


**Figure 2.4** Homepage of the ECARUCA website.

In addition to the public pages, the following restricted options on the ECARUCA website are available to professional account holders:

- Submit cytogenetic and molecular information.
- Submit clinical information.
- Search by chromosomal aberration.
- Search by clinical feature(s).
- List of all cases submitted by the centre of the account holder.
- List of all participating centres.
- Detailed Case of the Month information.

Furthermore, account holders receive an overview by email of all cases submitted to the ECARUCA database in the past month, thereby providing up-to-date clinical information and the possibility for publications together with colleagues.

A unique feature of the website is the restricted page for parents of children whose data has been submitted to the ECARUCA database. A case-specific parent account is created for all

newly submitted cases and sent to the referring clinician. With this account parents have access to the information of their own child in the database and can directly send follow-up information to the Clinical Database Managers.

## Submission of cases

Submission of new cases takes place through a clear online process. This process is composed of the submission of cytogenetic and/or molecular data and, subsequently, the submission of accompanying clinical data.

All new cases are presented to ECARUCA by entering the required cytogenetic and/or molecular information (Figure 2.5a). Data that are required include the karyotype, the exact breakpoints, ISCN quality and, if performed, the outcome of molecular techniques used. Furthermore, the name and email address of the clinician involved should be reported in case the cytogenetic submitter and the clinician are not the same person. The data submitted are checked by the Clinical Database Manager and, upon approval, the clinical data can be submitted by means of a separate data registration interface (Figure 2.5b). After entering all the data in the interface the user submits the information to the database with a single mouse click.

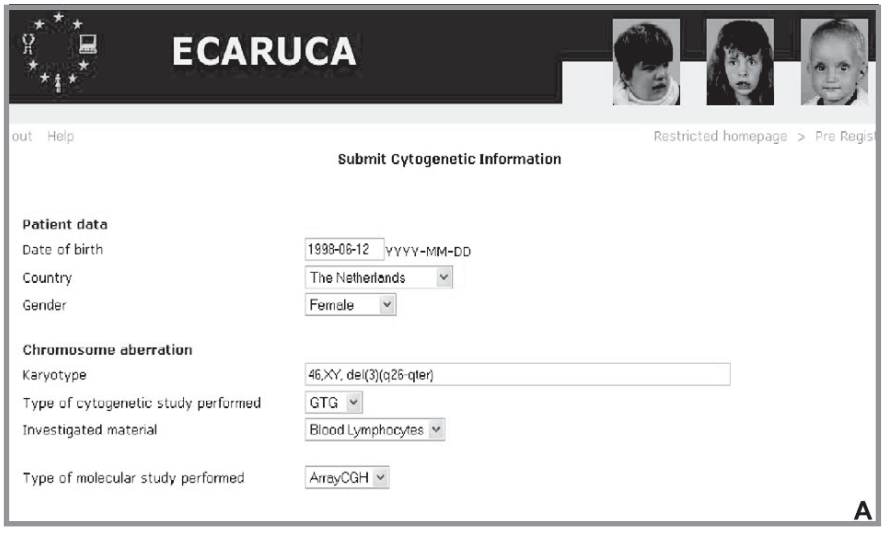


**Figure 2.5a** Submission of a new case starts with entering the required cytogenetic and/or molecular information.

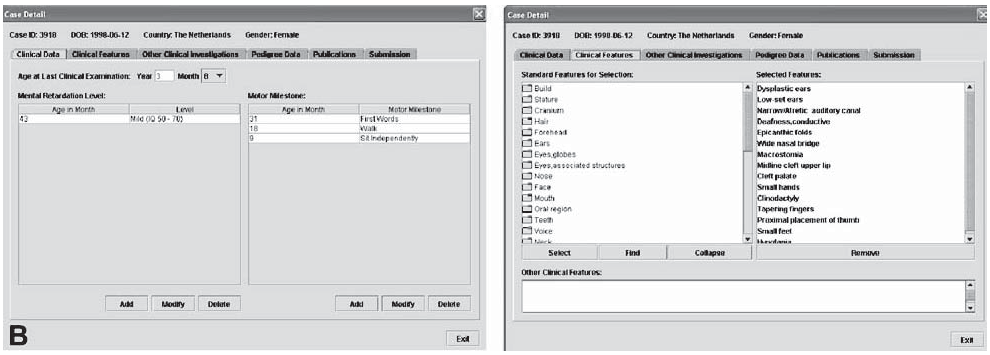


**Figure 2.5b** Secondly, the clinical data can be submitted by means of the clinical interface. The feature tree is used for selecting clinical features.

Any additional cytogenetic, molecular data and clinical follow-up information is submitted by email. The Clinical Database Manager carefully reviews all incoming data. Only when no further essential information is expected for a particular case and all data are confirmed to be certain, the case is made available for viewing by other account holders.

## Search functionalities

Account holders have access to query the database either by chromosome aberration or by clinical features according to a select list derived from the Winter–Baraitser London Dysmorphology DataBase. [15]

The option "Search by chromosomal aberration" allows the user to retrieve information about cases that concern a particular region on a chromosome of choice (Figure 2.6a). In the example, the search is specified to include all deletions in the region between the bands q21.3 and qter of chromosome 18. The search result provides not only cases for which the aberration is located within the boundaries of the chosen chromosome bands, but also those, which overlap the specified region. The user will see a list of all aberrations present in the database that are present within the region of interest (Figure 2.6b). By clicking on the aberration of interest, all features that accompany that aberration are listed. The final result (Figure 2.6c) is an overview of major and other clinical features of the total number of cases of that specific aberration. Also, information on average age at last examination and IQ are given together with a list of relevant publications.

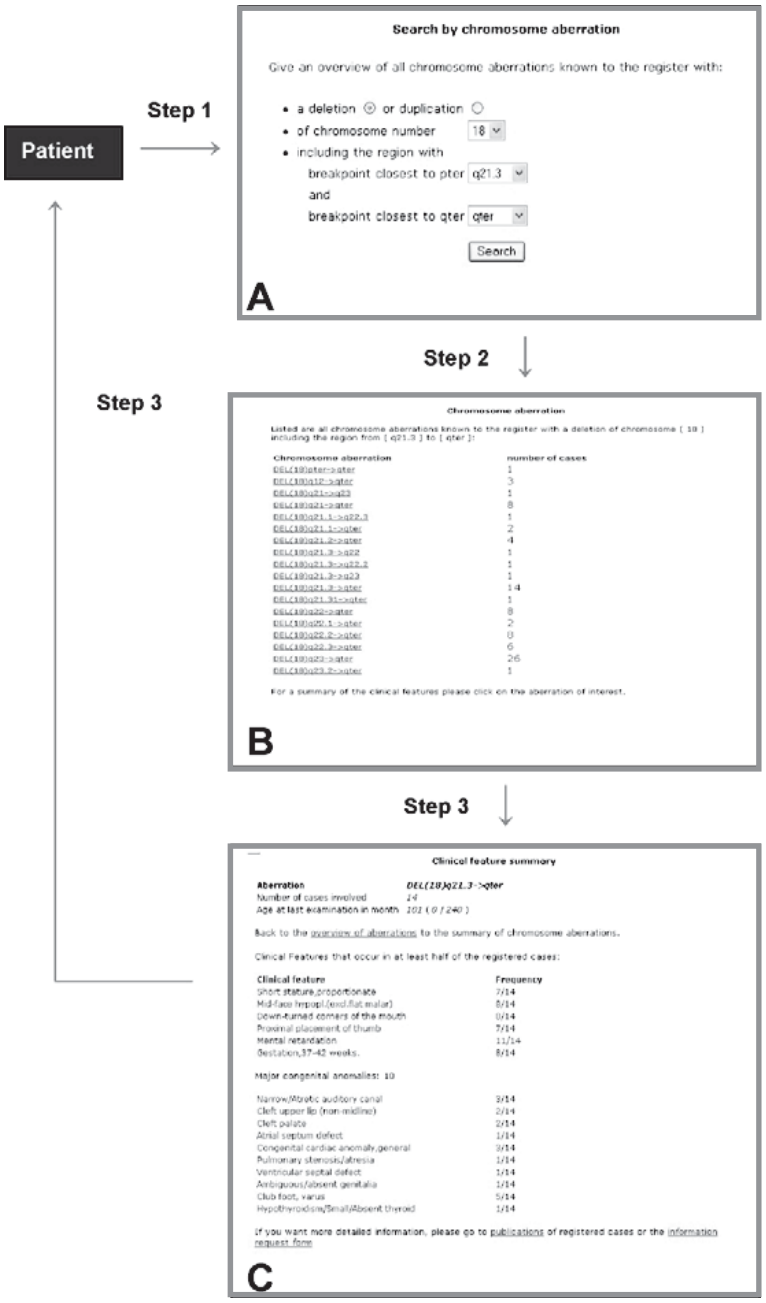


**Figure 2.6** An example of how to use the option "Search by chromosomal aberration", in which the physician/account holder is interested in all deletions in the region between the bands q21.3 and qter of chromosome 18 **(A)**. The search results in a list of aberrations **(B)**. The user selects the most suitable aberration to view the associated clinical features **(C)**. These clinical features can be used to provide advice to the patient concerned (Step 3).

The option "Search by clinical feature(s)" allows the user to find all chromosomal aberrations in the database that are associated with specified features. An example for the features platelet abnormalities, ptosis and ventricular septal defect is shown in Figure 2.7. The clinical features can be selected from a user-friendly expandable selection "tree", which is based on the feature list present in the widely known Winter–Baraitser London Dysmorphology DataBase. The User needs to indicate how many of the chosen features are minimally required to be associated with an aberration for it to be included in the search results. As a result, all types of aberrations for which patients display these clinical features are shown. This search strategy can provide a possible diagnosis and can in addition help to identify chromosomal regions and candidate genes for specific feature(s).

In case a professional has specific interest in a particular aberration, he or she can contact the Clinical Database Manager in order to receive more detailed information on an individual case.

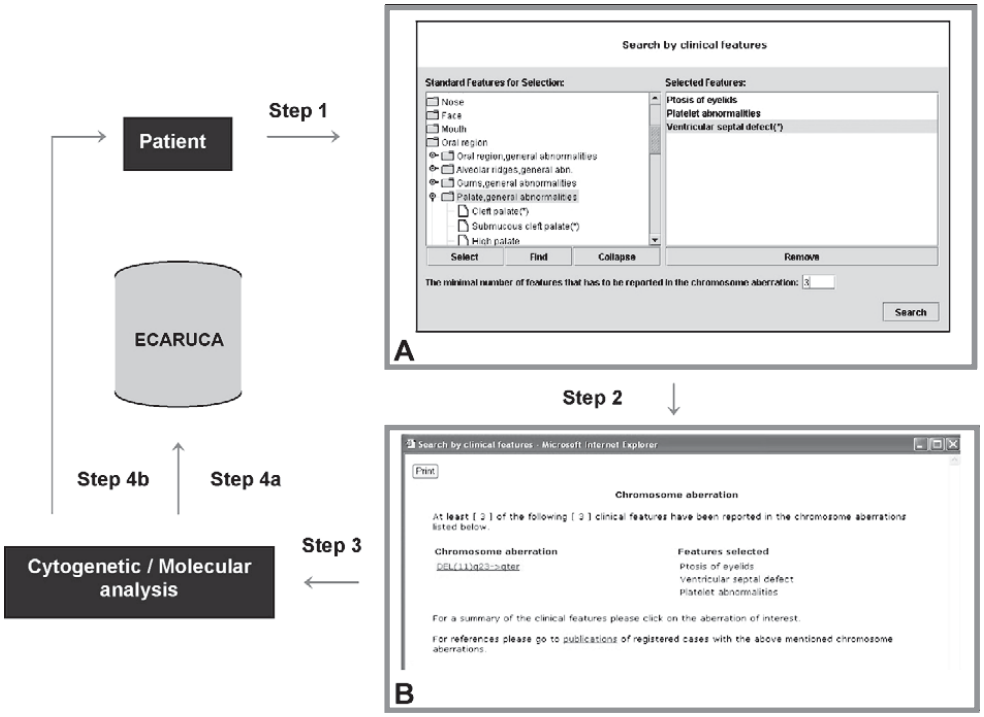


**Figure 2.7** An example of how to use the option "Search by clinical features", in which the account holder is interested in all cases which display the following three features as present in a patient: ptosis, platelet abnormalities and ventricular septal defect **(A)**. The search yields a number of aberrations in which patients display the selected features **(B)**. This result can be used as input for further genetic analysis (Step 3). The obtained laboratory result is submitted to ECARUCA (Step 4a) and sent to the physician in attendance (Step 4b).

## Cytogenetic verification

Another important component of the ECARUCA project is a collaboration between centres in Europe and the Mediterranean that provide cytogenetic, molecular and, if needed, clinical diagnostics on cases with a rare chromosomal aberration or cases with incomplete investigations.

In a number of genetic centres, especially in Eastern Europe, the financial or technical possibilities to perform high quality cytogenetic analyses are limited. As a consequence, breakpoints cannot be determined accurately. Because ECARUCA aims to be a reliable, high-standard database, these cases cannot be entered into the database without additional studies, resulting in a loss of a potentially high volume of interesting data. This may subsequently contribute to the growing gap between laboratories in Western Europe and those in the less developed countries.

In order to prevent the issue outlined above, less facilitated centres can receive assistance with the verification of cytogenetic data. Material can be sent to the Institute of Medical Genetics in Zurich, where the exact breakpoints will be determined. Subsequently, the case will be entered in the ECARUCA database.

In the year 2004, the samples of 57 patients originating from 10 different countries have been investigated. Techniques used include routine karyotyping, reverse painting, micro satellite analysis, chromosome micro dissection, FISH and array CGH. For most of these cases, the advanced cytogenetic analysis with high-resolution techniques resulted in a more precise delineation of the breakpoints. Subsequently, in a number of cases the initial designation of the breakpoints had to be revised, thereby having implications for karyotype–phenotype correlation. [16,17]

The correct determination of the chromosome aberration is not only important for the reliability of the ECARUCA database, but also has implications for clinical, counselling and scientific research activities.

# DISCUSSION

## Future aims

The main feature of the ECARUCA database is its interactivity: users can query the database, and at the same time they can submit cases in order to increase the number of cases in the database, making it more powerful and informative. Submitting an unpublished case to the database will create a platform for such rare cases to be viewed by colleagues who might themselves have observed a similar case, leading to joint publications.

At present, almost 4000 patients are collected in the database. In Europe alone, an annual number of 3000–5000 children carrying a rare chromosome aberration are born. Therefore it is expected that a considerable number of new cases coming from all over the world will be submitted to the database each year. Furthermore, by reviewing the medical literature on case reports and other publications, a supplemental number can be included every year.

The functionality of the ECARUCA database and the website are constantly being improved to meet the needs of its users. We try to make the submission procedure as complete as possible and simultaneously less time consuming. Furthermore, the adaptation of the database regarding the implementation of a search and entering facility at base pair level will ensure that the ECARUCA database can accommodate future techniques that have much higher resolution than that of the routine cytogenetic techniques presently used. New high-tech methods like array CGH are currently used in a limited number of laboratories, but the general expectation is that this technique will become a standard diagnostic method in the near future.

The possibility of collecting aberrations at the level of base pairs will have positive implications for the scientific use of the ECARUCA database. First of all, it will become easier for non-clinical professionals to interpret and use the available data. Secondly, scientists, notably in the field of molecular genetics, require a standardised way of storing data at the submicroscopic level, which will be provided by ECARUCA. Storing of molecular data generated by high quality experiments in a standardised format greatly enhances the international search for new genes.

The easy and free access to the database for doctors and scientists is a good basis for collaboration in genotype–phenotype studies. The fast progress in the development of new techniques provides an increasing knowledge on genetic abnormalities. However, only by

understanding the clinical consequences of these genetic changes can progress be made towards gene identification and ultimately perhaps diagnostic interventions.

A limited other Internet sources are available to look for information on rare chromosome aberrations, e.g. DECIPHER and the Mendelian Cytogenetics Network. However, the first database only collects submicroscopic aberrations while the second one collects and provides information on balanced chromosomal rearrangements. Therefore, the ECARUCA database complies with the need to fill this gap.



In the future, ECARUCA will also focus on the follow-up of patients present in the database. This will give physicians more insight into how their patient will most likely develop and whether there are any specific new motor skills or physical abnormalities that need special attention.

In addition to the expected increase in knowledge among professionals, we wish to develop correct, understandable and clear information for parents and other interested parties. This should comprise a textual summary of the main clinical and developmental aspects of the aberrations included in the database. Since it would be impossible to include a description of all aberrations, we will restrict the information to a certain extension of chromosome regions or to more frequently arising small aberrations. The physician in attendance can retrieve more specific information that matches the exact deleted or duplicated region in the patient from the database.

## Concluding remarks

The Internet era has given rise to all kinds of new possibilities in information processing. The Internet is especially helpful in finding information about unusual matters like rare chromosome aberrations. Until the existence of ECARUCA, however, an Internet search on a particular rare chromosome disorder usually yielded only a couple of websites with a limited amount of information, sometimes out-of-date and never including follow-up data of patients.

Although professionals have the opportunity to look up information in medical journals, books and other sources, these have the limitation, except for review articles, that only one or two patients are described independently and not the frequency of clinical features occurring in a group of patients with the same aberration. Furthermore, follow-up data of published patients is hard to retrieve.

Altogether, ECARUCA can be considered to be a new system that meets the needs of scientists, physicians and patients and their family members involved with rare chromosomal aberrations, in an interactive way.

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chapter 3.1

# Balanced into array: genome-wide array analysis in 54 patients with an apparently balanced *de novo* chromosome rearrangement and a meta-analysis

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## ABSTRACT

High-resolution genome-wide array analysis enables detailed screening for cryptic and submicroscopic imbalances of microscopically balanced *de novo* rearrangements in patients with developmental delay and/or congenital abnormalities.

In this report, we added the results of genome-wide array analysis in 54 patients to data on 117 patients from seven other studies. A chromosome imbalance was detected in 37% of all patients with two-breakpoint rearrangements. In 49% of these patients, the imbalances were located in one or both breakpoint regions. Imbalances were more frequently (90%) found in complex rearrangements, with the majority (81%) having deletions in the breakpoint regions. The size of our own cohort enabled us to relate the presence of an imbalance to the clinical features of the patients by using a scoring system, the De Vries criteria, that indicates the complexity of the phenotype. The median De Vries score was significantly higher ($P$=0.002) in those patients with an imbalance (5, range 1-9) than in patients with a normal array result (3, range 0-7). This study provides accurate percentages of cryptic imbalances that can be detected by genome-wide array analysis in simple and complex *de novo* microscopically balanced chromosome rearrangements and confirms that these imbalances are more likely to occur in patients with a complex phenotype.

## INTRODUCTION

The estimated frequency of balanced chromosome rearrangements in a population of unselected newborns is 0.52%. [1] The majority of these translocations, insertions and inversions is transmitted from one of the parents and not associated with abnormal phenotypes. [2] In 1991, Warburton [3] reported data on the frequency and outcome of cases with apparently balanced, *de novo*, rearrangements detected at amniocentesis in over 350 000 pregnancies. She found that a microscopically balanced, *de novo*, reciprocal translocation was detected in 1 out of every 2000 pregnancies. The frequency of congenital abnormalities in fetuses and newborns with *de novo*, reciprocal translocations or inversions has been estimated at 6.1 and 9.4%, respectively. [3] This is more than twice as high as the risk of 2-3% in the general population.



The increased number of abnormal phenotypes can be caused by:
(1) a microdeletion or microduplication at the translocation or inversion breakpoint(s) which is only detectable by high-resolution techniques, (2) disruption or modulation of the expression of gene(s) located at the breakpoint(s) and (3) otherwise inactivation (position effect) of gene(s) at the breakpoint region(s). Thus, an apparently balanced, *de novo*, chromosome rearrangement can underlie an abnormal phenotype, but it may also be coincidental. The actual confirmation or rejection of causality by detecting a cryptic deletion or duplication at the assumed breakpoints or elsewhere in the genome is often lacking. The unbalanced nature of small rearrangements will most often escape detection, as the resolution of standard cytogenetic banding techniques is only 5-10 Mb. It has already been shown that the yield of chromosome abnormalities in patients with developmental delay (DD) and/or multiple congenital anomalies (MCA) increases considerably with the resolution of the technique used. A microscopically visible chromosome abnormality can be detected by routine karyotyping in 3-5% of all DD/MCA patients, excluding Down's syndrome, [4-6] whereas genome-wide array-based techniques are able to detect a chromosome imbalance in up to 15-20% of such cases. [5,7,8]

Recent studies have reported on genome-wide array analysis used to identify cryptic imbalances in cohorts of DD/MCA patients with an apparently balanced, *de novo*, chromosome rearrangement (Table 3.1.1). [9-15] A cryptic imbalance was detected by genome-wide array analysis in 33-100% of DD/MCA patients with a *de novo* chromosome rearrangement. In the majority of patients, the imbalance was detected at one or more breakpoints, although a large percentage of imbalances (15-40%) was found elsewhere in the genome. The frequency of detected imbalances is significantly higher in patients with a

**Table 3.1.1** Overview of genome-wide array studies in patients with apparently balanced, *de novo* chromosome rearrangements and abnormal phenotypes

| Study | Gribble *et al.* [9] | De Gregori *et al.* [10] | Sismani *et al.* [11,a] | Baptista *et al.* [12,a,b] | Higgins *et al.* [13,a] | Schluth-Bolard *et al.* [14,a] | Gijsbers *et al.* [15,a] | Present study | Total |
|---|---|---|---|---|---|---|---|---|---|
| Array platform | 1 Mb BAC | 44k or 244k oligo Agilent | 1 Mb Cytochip Bluegnome | Sanger 30k Whole Genome Tilepath | 2600 BAC Spectral Genomics or 244k oligo Agilent | 44k or 244k oligo Agilent | Affymetrix GeneChip 250k Nspl | 32k BAC, 105k or 244k oligo Agilent or 250k SNP Affymetrix | |
| Patients with 2 bp rearrangement[c] | 8 | 27 | 6 | 12 | 10 | 28 | 5 | 46 | 142 |
| Patients (percentage) with imbalance at breakpoint | 0 (0%) | 7 (26%) | 0 (0%) | 3 (25%) | 2 (20%) | 6 (21%) | 3 (60%) | 5 (11%) | 26 (18%) |
| Patients (percentage) with genomic imbalance elsewhere | 3 (38%) | 4 (15%) | 2 (33%) | 1 (8%) | 3 (30%) | 6 (21%) | 2 (40%) | 6 (13%) | 27 (19%) |
| Patients with CCR[c] | 2 | 13 | 0 | 1 | 0 | 5 | 0 | 8 | 29 |
| Patients (percentage) with imbalance at breakpoint | 2 (100%) | 9 (69%) | 0 (0%) | 0 (0%) | 0 (0%) | 4 (80%) | 0 (0%) | 6 (75%)[d] | 21 (72%) |

| Study | Gribble *et al.* [9] | De Gregori *et al.* [10] | Sismani *et al.* [11,a] | Baptista *et al.* [12,a,b] | Higgins *et al.* [13,a] | Schluth-Bolard *et al.* [14,a] | Gijsbers *et al.* [15,a] | Present study | Total |
|---|---|---|---|---|---|---|---|---|---|
| Patients (percentage) with genomic imbalance elsewhere | 0 (0%) | 3 (23%) | 0 (0%) | 1 (100%) | 0 (0%) | 1 (20%) | 0 (0%) | 1 (13%)[d] | 6 (21%) |
| Total number of patients | 10 | 40 | 6 | 13 | 10 | 33 | 5 | 54 | 171 |
| Patients (percentage) with imbalance at breakpoint | 2 (20%) | 16 (40%) | 0 (0%) | 3 (23%) | 2 (20%) | 10 (30%) | 3 (60%) | 11 (20%) | 47 (27%) |
| Patients (percentage) with genomic imbalance elsewhere | 3 (30%) | 7 (18%) | 2 (33%) | 2 (15%) | 3 (30%) | 7 (21%) | 2 (40%) | 7 (13%) | 33 (19%) |
| Total number (percentage) of patients with an imbalance | 5 (50%) | 23 (58%) | 2 (33%) | 5 (38%) | 5 (50%) | 17 (52%) | 5 (100%) | 17 (31%)[d] | 79 (46%)[d] |

[a] Only patients with a *de novo* cytogenetically balanced rearrangement, studied by array CGH have been included in this table.
[b] Including two patients with premature ovarian failure and two patients with severe oligospermia and no additional known abnormalities.
[c] 2 bp = Two-breakpoint rearrangement at routine karyotyping; CCR = complex chromosomal rearrangement (three or more breakpoints) at routine karyotyping.
[d] One patient with a CCR had imbalances at a breakpoint and elsewhere, therefore the total of imbalances at breakpoints and elsewhere equals -1.

more complex chromosome rearrangement (CCR), involving more than two chromosomes and/or more than two breakpoints. [9,10,14] In all studies, the reported imbalances were assumed to cause the abnormal phenotype.

In contrast to the studies performed in DD/MCA patients, Baptista *et al.* [12,16] compared a cohort of 31 phenotypically normal individuals carrying a balanced chromosome rearrangement with a cohort of 14 DD/MCA patients. No genomic imbalances at the breakpoints, or elsewhere in the genome, were detected in the 31 normal carriers, whereas a disease-causing imbalance was detected in 4 out of 14 DD/MCA patients. The authors concluded that translocations in patients with a clinically abnormal phenotype are molecularly distinct from those in normal individuals. An unexpected finding was that the frequency of gene disruption due to a chromosome rearrangement did not differ between phenotypically abnormal patients and the normal study population. [12] However, the percentage of disrupted genes that have a role in the nervous system was higher in the phenotypically abnormal patients.

Since there is limited data on patients with apparently balanced chromosome rearrangements, we decided to evaluate the results obtained from genome-wide array analysis in a cohort of 54 DD/MCA patients and a cytogenetically balanced, *de novo*, chromosome rearrangement. Since this is the largest postnatal cohort of DD/MCA patients with *de novo* balanced rearrangements reported thus far, we were able to improve the estimated percentage of submicroscopic imbalances detected by genome-wide array analysis in *de novo* chromosome rearrangements. The size of the cohort also enabled us to relate the probability of finding an imbalance to the clinical phenotype of the patient by using the De Vries scoring system. [17]

## PATIENTS AND METHODS

### Patient selection

Clinical data and samples were collected from 54 patients with an apparently balanced, *de novo*, structural chromosome rearrangement. All patients had been referred for karyotyping because of DD and/or MCA and were enrolled in the study for diagnostic purposes. All chromosome rearrangements were detected by routine cytogenetic analysis at a minimum band level of 500: 46 patients carried a two-breakpoint rearrangement; 40 patients had a reciprocal translocation, while 6 patients carried an inversion. Eight patients had a CCR with at least three breakpoints.

All patients, parents or legal representatives gave informed consent for this study, according to local guidelines.

## Collection of clinical data

Clinical data were derived from medical records using a standardized form. Additional information was requested from the referring clinicians whenever necessary. All patients were scored according to adapted De Vries criteria, which provided a checklist for patients with submicroscopic subtelomeric rearrangements (Table 3.1.2). [17] Family history was replaced by DD in this scoring system, because a positive family history, either compatible or non-compatible with Mendelian inheritance, does not enhance the chance of finding imbalances in the breakpoint regions in patients with a *de novo* chromosome rearrangement.



In contrast, the De Vries criteria were developed for patients with an intellectual disability, while not all the patients in our study had a DD. Therefore, one and two points were given for mild-to-moderate and severe DD, respectively. Severe DD was defined as a Developmental Quotient <30, while mild-to-moderate DD was a Developmental Quotient between 30 and 70. In this way, the maximum number of points that could be scored remained 10 (Table 3.1.2).

**Table 3.1.2** De Vries score and adjusted De Vries score for assessing clinical phenotypes

| Original De Vries score [17] | | De Vries score, adjusted for this study | |
|---|---|---|---|
| Trait (points) | Score | Trait (points) | Score |
| Family history of MR<br>Compatible with Mendelian inheritance (1)<br>Incompatible with Mendelian inheritance (2) [a] | 1-2 | Developmental delay<br>Mild-moderate developmental delay (1)<br>Severe developmental delay (2) | 1-2 |
| Prenatal onset of growth retardation | 2 | Prenatal onset of growth retardation | 2 |
| Postnatal growth abnormalities<br>Microcephaly (1)<br>Short stature (1)<br>Macrocephaly (1)<br>Tall stature (1) | Max 2 | Postnatal growth abnormalities<br>Microcephaly (1)<br>Short stature (1)<br>Macrocephaly (1)<br>Tall stature (1) | Max 2 |
| ≥ 2 Facial dysmorphic features [b] | 2 | ≥ 2 Facial dysmorphic features [b] | 2 |
| Non-facial dysmorphism and congenital abnormalities [c] | 1-2 | Non-facial dysmorphism and congenital abnormalities [c] | 1-2 |
| Total maximum | 10 | Total maximum | 10 |

[a] Including discordant phenotypes.
[b] Notably, hypertelorism, nasal anomalies and ear anomalies.
[c] Notably, hand anomaly, heart anomaly, hypospadias with/without undescended testis; assign 1 point for each, with a maximum score of 2 points.

## Genome-wide array analysis

Array analysis with an average genome-wide resolution of ~200 kb was performed using either an Agilent 105k or 244k oligo array, a 32k BAC array as previously described, [18] or the Affymetrix 250k SNP array platform, [19] following the protocols provided by the manufacturers (Agilent Technologies and Affymetrix Inc., Santa Clara, CA, USA).

For the Agilent array reference DNA, a mixture of 40 male or female DNA samples of the same gender was used as control. The data were processed using Feature Extraction V.9.1 and CGH analytics V.3.4.27 provided by the manufacturer (Agilent Technologies). For the SNP array experiments, copy number estimates were determined using the updated version 2.0 of the CNAG (Copy Number Analyzer for Affymetrix GeneChip mapping) software package. [20] The normalized ratios were then analyzed for genomic imbalances by a standard Hidden Markov Model, essentially as described before. [18] The SNP array data obtained from patient DNA were compared with SNP array data from 10 healthy, sex-matched individuals.

Regardless of the array platform employed, genome-wide data analysis was performed using previously determined criteria which provide 95% confidence of representing a true copy number variation (CNV). [21] A CNV was considered significant if five or more consecutive probes showed a single copy number loss ($n$=1), or at least seven consecutive SNPs showed a single copy number gain ($n$=3) for the Affymetrix array, or four or more consecutive probes showed gains or losses for the Agilent array. For interpretation purposes, various public web sources were consulted, including the Online Mendelian Inheritance of Man (http://www.ncbi.nlm.nih.gov/Omim), the DECIPHER database (http://decipher.sanger.ac.uk) and ECARUCA (http://www.ecaruca.net). A CNV was considered a normal genomic variant if it had been detected in at least three control individuals as reported in the Database of Genomic Variants (http://projects.tcag.ca/variants), and/or been encountered in at least three in-house control samples. Data analyses were based on the NCBI36/hg18 build of the human genome.

## Fluorescent in situ hybridization analysis

To validate the gains or losses identified by genome-wide array analysis, region-specific fluorescent in situ hybridization (FISH) was performed following routine protocols. Bacterial Artificial Chromosome (BAC) clones were selected from the human library RPCI-11 according to the UCSC Human Genome Assembly (freeze March 2006) and kindly provided by the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk) or obtained from the 32k

set of BAC DNAs in the Nijmegen laboratory. BAC DNA was indirectly labeled with biotin- or digoxigenin-11-dUTP using Nick translation. Slides were hybridized overnight at 37 °C and fluorescently labeled with FITC or Texas Red.

### Multiplex ligation-dependent probe amplification.

To validate the gains identified by array analysis, region-specific multiplex ligation-dependent probe amplification (MLPA) was performed. For each region, two uniquely sized probes were developed in accordance with a protocol provided by MRC Holland (Amsterdam, the Netherlands). Ten probes were combined in one MLPA assay together with a DNA quantity and a DNA denaturation control mix (EK-1 kit, MRC Holland). The procedure was further carried out as described by De Vries *et al.* [18]



## RESULTS

In this study, 54 patients with an apparently balanced, *de novo* chromosome rearrangement and an abnormal clinical phenotype were analyzed for submicroscopic chromosome imbalances by genome-wide array analysis. Forty-six patients had a two-breakpoint rearrangement upon routine karyotyping. In eight patients, a more complex aberration was found. All patients had facial dysmorphisms and/or congenital malformations and 46 out of 52 patients (88%) showed DD, varying from mild psychomotor retardation and speech delay to severe DD. Development could not be assessed in two patients because they died at the age of 1 day and 2 months, respectively (patients 12 and 43). A detailed description of all the phenotypes is presented in Table 3.1.3.

The total number of CNVs, including well-known benign CNVs, detected by the platforms used ranged from 2 to 12 with an average of 5.6 per patient (Table 3.1.3). All the potentially causative, copy number alterations detected by array could be confirmed by FISH (losses), MLPA (gains) or an independent array platform.

### CNVs at or near the breakpoint regions

In 11 out of 54 patients (20%), the apparently balanced rearrangement was found to be unbalanced at the breakpoint region(s). We found no gains but 16 losses in these 11 patients in total (Table 3.1.4A). The size of the losses varied from 0.1 to 15.3 Mb. Seven patients had a

**Table 3.1.3** Overview of all patients giving karyotype, phenotype and De Vries score

| Patient | Karyotype[a] | Array Platform[b] | No. of CNVs[c] | Phenotype[d] | Adjusted De Vries score[e] |
|---|---|---|---|---|---|
| Two-breakpoint aberrations | | | | | |
| 1 | t(X;3)(p21.3;p25.1) | 32k | 3 | Dolichocephaly, long face, strabism, full tip of the nose, prominent columella, dysmorphic ears, short philtrum, hyperthyroidism, severe DD, speech delay, convulsions, hypotonia | 4 |
| 2 | t(X;10)(p22.32;q22.2) | 244k | 4 | Short stature, microcornea, iris coloboma, cataract, short and broad hand and feet, hirsutism, adipositas, secundary amenorrhoea, severe DD, blindness, hypotonia | 4 |
| 3 [23, pt A] | t(X;19)(p11.4;q13.3) | 32k | 6 | Autism, borderline DD (IQ 82), mild hypotonia | 0 |
| 4 | inv(X)(q22.1q28) | 32k | 7 | Down-slant palpebral fissures, open mouth appearance, pes planus, convulsions, speech delay, autism | 2 |
| 5 | t(1;2)(p35;q33) | 105k | 4 | Chondrodysplasia punctata, severe short stature, low-set dysplastic ears, flat nose, hemangioma, PMR, deafness, hypertonia | 5 |
| 6 | t(1;6)(p22.1;q15) | 244k | 7 | Macrocephaly, cerebral atrophy, deep-set eyes, prominent fore head, midface hypoplasia, low nasal bridge, short philtrum, low-set ears, pectus excavatum, small hands with broad short phalanges of the thumbs, pes planus, severe DD | 6 |
| 7 | t(1;8)(p22.1;p23.3) | 244k | 6 | Severe DD, absent tendon reflexes, autism, hypotonia | 2 |
| 8 | t(1;14)(q42.1;q31.1) | 32k | 7 | Dysplastic ears, mild DD, obstipation | 2 |
| 9 | t(1;16)(q21;p11.2) | 250k | 6 | Pre-auricular tag, DD (IQ 50), behavioral and sleep problems | 2 |
| 10 | t(1;17)(p36.1;q11) | 244k | 5 | Mild DD, obstipation, recurrent airway infections | 1 |
| 11 | inv(1)(p22.3p34.1) | 32k | 4 | Macrocephaly (+4.5 SD), dolichocephaly, mild ventriculomegaly, hypertelorism, upward slanted and narrow palpebral fissures, micrognathia, proximally placed thumbs, mild DD | 5 |
| 12 | t(2;9)(q34;p22) | 244k | 2 | Broad tip of the nose, micrognathia, single palmar crease, convulsions, deceased at age 2 months | NA |
| 13 | t(2;10)(p13;p14) | 105k | 3 | Broad tip of the nose, moderate/severe DD, convulsions, hypotonia | 2 |
| 14 [24] | t(2;10)(p23;q22.1) | 32k | 6 | Birth weight >P98, macrocephaly, sparse hair, hypoplastic alae nasi, dysplastic ears, moderate DD, psychotic disorder, hypotonia, nasal speech, disturbed serine metabolism | 4 |
| 15 | t(2;10)(p25;q26) | 250k | 5 | Epicanthus, club foot, hyperlaxity, mild DD (IQ 64), affective psychotic episodes | 2 |
| 16 | t(2;10)(q22;q22.3) | 244k | 10 | Growth retardation, down-slanting palpebral fissures, small nose, mild/moderate DD, convulsions, hypotonia, obstipation | 4 |

| Patient | Karyotype[a] | Array Platform[b] | No. of CNVs[c] | Phenotype[d] | Adjusted De Vries score[e] |
|---|---|---|---|---|---|
| Two-breakpoint aberrations | | | | | |
| 17 | t(2;10)(q23;p12) | 244k | 8 | Narrow fore head, high narrow palate, mild retrognathia, mild DD, autism | 3 |
| 18 [25] | t(2;14)(q37.3;q13) | 105k | 2 | IUGR, microcephaly, iris coloboma, laryngomalacia, umbilical hernia, inguinal hernia, severe PMR | 6 |
| 19 | t(2;15)(p22.2;p11) | 32k | 3 | Left-sided hemiparesis, upturned nose, 3 maxillary incisors, absent lower canine tooth, dilatation of aorta, scoliosis, arachnodactyly, mild DD, pubertas tarda, hyperlaxity | 5 |
| 20 | t(2;17)(p25;q23) | 250k | 3 | No dysmorphisms, eczema, PMR, speech delay, IQ 50-60 | 1 |
| 21 | t(2;18)(q23;q23) | 250k | 6 | Macrosomia at birth, bulbous nose, high narrow palate, pointed chin, tibial bowing, obesity, mild DD | 4 |
| 22 | inv(2)(q11.2q33) | 32k | 5 | High birth weight (>P98), deep-set eyes, short palpebral fissures, high bridge of the nose, micrognathia, high palate, micropenis, large hands, mild DD, aggressive behavior | 4 |
| 23 | t(3;12)(p13;p13.3) | 244k | 4 | Macrocephaly, macro-orchidism, nervus opticus atrophy, kyphosis, DD | 4 |
| 24 | t(4;8)(p16.1;p23.1) | 244k | 3 | Short stature, pre-auricular tags, synophris, prognathia, epicanthus, broad nasal bridge, thin upper lip, wide spaced teeth, mild DD, behavioral problems | 4 |
| 25 | t(4;12)(p12;q13.2~13.3) | 244k | 3 | Hypertelorism, large ears, broad tip of the nose, short philtrum, thin upper lip, recurrent infections, no DD | 2 |
| 26 | t(4;16)(q33;q12.2) | 244k | 8 | Microcephaly, moderate/severe DD, hypotonia | 3 |
| 27 | t(4;17)(q23;q21) | 244k | 4 | Short stature (<P3), cerebral atrophy, strabism, scoliosis, pes plani, severe DD, autism, hypotonia | 4 |
| 28 | t(5;7)(p15.1;p22) | 32k | 5 | Upturned nose, mild DD, speech delay, autism, obsessive eating disorder | 2 |
| 29 | t(5;10)(q33;q25) | 250k | 4 | High birth weight (P98), blepharophimosis, epicanthus, strabism, long face, prominent nose, aplasia of nails, hip dysplasia, obesity, sensorineural deafness, severe DD, hypotonia | 5 |
| 30 | t(5;17)(p15.3;q25.3) | 32k | 6 | Macrocephaly, dolichocephaly, mild hypertelorism, epicanthus, short philtrum, micrognathia, overriding 2nd and 4th toes, mild conductive hearing loss, severe DD, speech delay, mild hypotonia | 6 |
| 31 | inv(5)(q14q33) | 32k | 2 | Protruding tongue, down-slanting palpebral fissures, strabism, posteriorly rotated ears, hirsutism, moderate DD, speech delay, autistic spectrum disorder | 3 |

*Table 3.1.3 continues on next page*

**Table 3.1.3** *Continued*

| Patient | Karyotype[a] | Array Platform[b] | No. of CNVs[c] | Phenotype[d] | Adjusted De Vries score[e] |
|---|---|---|---|---|---|
| Two-breakpoint aberrations | | | | | |
| 32 | t(6;9)(q21;p24) | 250k | 3 | Severe DD, no speech, convulsions, mild hypotonia | 2 |
| 33 | t(6;11)(p12.3;p14.2) | 32k | 10 | Macrocephaly, strabism, high palate, hypertrichosis lumbosacralis, cryptorchidism, camptylodactyly dig V, pes plani valgi and metatarsus adductus, moderate DD, speech delay, mild sensorineural deafness, mild hypotonia, | 5 |
| 34 | t(6;11)(q16.2;p15.1) | 250k | 5 | Epicanthus, severe DD, no speech, mild hypotonia, | 3 |
| 35 | inv(6)(p11.1q21) | 32k | 6 | Eye disorder, mild to moderate DD | 2 |
| 36 | t(7;15)(p14;p11.2 | 244k | 7 | Microcephaly, small nose, dysplastic ears, low-set left ear, clinodactyly, cryptorchidism, severe DD, West syndrome | 5 |
| 37 [26, pt 3] | inv(7)(p22q21.3) | 244k | 5 | Ectrodactyly of both hands and feet, atriovenous malformation of right hand, autism, no DD | 1 |
| 38 | t(8;14)(q21.2;q12) | 32k | 5 | Microcephaly, partial agenesis corpus callosum, deep-set eyes, strabism, high palate, open mouth appearance, scoliosis, pectus excavatum, short distal phalanges, severe DD, absent speech, convulsions, obstipation | 7 |
| 39 | t(10;16)(q24.1;p11.2) | 244k | 8 | Epicanthus, hypogonadism, obesity, mild DD, autism | 2 |
| 40 | t(12;14)(q13.1;q32.3) | 244k | 6 | Trigonocephaly (familial), mild dysplastic ears, no DD | 2 |
| 41 | t(12;14)(q24.1;q11.2) | 244k | 5 | Long narrow face, sparse hair, broad nasal bridge, umbilical hernia, scoliosis and asymmetrical thorax, mild DD, hypotonia with hypertonia of extremities | 3 |
| 42 [22, pt 8] | t(12;15)(q24.1;q21.1) | 244k | 9 | Marfan phenotype, broad nasal bridge, short philtrum, long and small fingers and toes, celiac disease, PMR, hypotonia, no intellectual disability | 3 |
| 43 | t(13;17)(q32;q21) | 32k | 4 | Deceased 1 day after uneventful pregnancy and birth, enlarged liver, steatosis | NA |
| 44 | t(17;22)(q23;q12.2) | 32k | 4 | Long narrow face, down-slanting palpebral fissures, retrognathia, severe scoliosis, pectus excavatum, arachnodactyly, short 4th metatarsals, hallux valgus, mild/moderate DD, cutis marmorata | 5 |
| 45 [27] | t(18 ;20)(q21.1 ;q11.2) | 32k | 12 | Broad, square face, high narrow palate, bilateral single palmar crease, pes planus, mild DD | 3 |
| 46 | t(19;21)(q13.3;q22.3) | 244k | 5 | Microcephaly, epicanthus, high nasal bridge, overfolded helices, valvular pulmonary stenosis, pectus excavatum, mild webbing of the fingers, delayed speech development | 6 |

| Patient | Karyotype[a] | Array Platform[b] | No. of CNVs[c] | Phenotype[d] | Adjusted De Vries score[e] |
|---|---|---|---|---|---|
| Complex chromosome rearrangements | | | | | |
| 47 | ins(5;17)(pter;p13.33p13.1) | 32k | 9 | Short stature (<P3), microcephaly, broad nasal bridge, thin upper lip, ASD, mild DD, persistent diarrhea | 6 |
| 48 | ins(1;11)(p22;q23.1q24.3) inv(1)(p13q23) | 32k | 2 | Short stature, mild trigonocephaly, epicanthus, upslant palpebral fissures, short nose, hearing loss, carp mouth, short 4th metatarsal bone left, pes planus, moderate DD, convulsions, obesity | 5 |
| 49 | der(2)ins(8;2)(q2?;p15p21), der(8)ins(8;2)inv(p?;q?) | 32k | 4 | Short stature (<P3), microcephaly, epicanthus, dysplastic ears, carp mouth, severe gastro-oesophageal reflux, kyphoscoliosis, contractures, rocker bottom feet, severe DD, absent speech, mild hypotonia | 8 |
| 50 | der(2),der(10),der(18) | 250k | 4 | Microcephaly, severe PMR, hypotonia | 3 |
| 51 | der(6)t(6;9)(p21.3;q22) ins(6;13)(p21.3;q?21q31), der(9)t(6;9),der(13)ins(6;13) | 32k | 5 | Birth weight <P3, short stature (<P3), microcephaly, enlarged 4th ventricle, ptosis, strabism, broad high nasal bridge, low dysplastic ears, cleft palate, micrognathia, ASD, clinodactyly dig V, severe speech delay, hypotonia, compulsive behaviour, obstipation | 9 |
| 52 | t(10;18;14) (p15.3;q12.2;q32.3) | 244k | 6 | Sotos syndrome (NSD1 mutation), height and head circumference >P99, broad high forehead, hypertelorism, broad nasal bridge, overfolded helices, pectus excavatum, mild DD, autism | NA |
| 53 [28] | der(2),der(3),der(7),der(11) | 32k | 7 | Hypertelorism, everted large nose, full lips, pectus carinatum, short fingers, convulsions, severe DD, absent speech | 5 |
| 54 | t(2;6;12;3) (q24;q23;q12;p13) | 250k | 5 | PMR, hypotonia | 1 |

[a] Based on conventional karyotype and FISH analysis.
[b] 32k=32k BAC array; 105k=Agilent 105k oligonucleotide array; 244k=Agilent 244k oligonucleotide array; 250k=Affymetrix 250k SNP array.
[c] The total number of copy number variations (CNVs) detected, including well-known benign CNVs. See Table 3.1.4 for potentially causative copy number alterations.
[d] ASD = atrial septal defect; DD = developmental delay; PMR = psychomotor retardation.
[e] See Table 3.1.2, NA = not applicable.

**Table 3.1.4** Summary of patients with an imbalance at the breakpoints regions (A) or elsewhere in the genome (B) detected by genome-wide array analysis

| Pt | Karyotype | Imbalance | Size (Mb)<br>Position (Mb) | *De novo*/<br>inherited | Clinical relevance[a] | Array platform | Confirmation method |
|---|---|---|---|---|---|---|---|
| A | At breakpoint region | | | | | | |
| 6 | t(1;6)(p22.1;q15) | del 1p21.3 (51 oligos)<br>del 6q14.1q15 (680 oligos) | 1.07, 95.81-96.88<br>9.21, 81.72-90.93 | *De novo*<br>*De novo* | No<br>Yes | 244k Agilent | FISH |
| 19 | t(2;15)(p22.2;p11) | del 2p22.3p22.1 (84 BACs) | 7.2, 32.8-40.0 | *De novo* | Yes | 32k BAC | FISH |
| 24 | t(4;8)(p16.1;p23.1) | del 4p16.3p16.1 (501 oligos) | 4.52, 4.33-8.85 | *De novo* | Yes | 244k Agilent | FISH |
| 34 | t(6;11)(q16.2;p15.1) | del 6q16.1q16.2 (57 oligos) | 0.66, 98.44-99.10 | *De novo* | Yes | 250k SNP | FISH |
| 42 [22] | t(12;15)(q24.1;q21.1) | del 15q21.1q21.2 (496 oligos) | 4.25, 46.12-50.37 | *De novo* | Yes | 244k Agilent | FISH |
| 47 | ins(5;17)(pter;p13.3p13.1) | del 17p13.3p13.2 (32 BACs) | 3.3, 2.8-6.2 | *De novo* | Yes | 32k BAC | FISH |
| 48 | ins(1;11)(p22;q23.1q24.3) inv(1)(p13q23) | del 1p22.1p13.3 (176 BACs) | 15.3, 94.2-109.5 | *De novo* | Yes | 32k BAC | None[b] |
| 49 | der(2)ins(8;2)(q2?;p15p21), der(8)ins(8;2)inv(p?;q?) | del(8)(q21.11) (7 BACs)<br>del(8)(q22.1) (11 BACs)<br>del(8)(q24.21) (26 BACs) | 0.7, 75.4-76.1<br>1.1, 94.2-95.3<br>2.4, 128.7-131.1 | <br><br>*De novo* | ?<br>?<br>Yes | 32k BAC | FISH |
| 50 | der(2)(10qter->10q2?5::2p?5::10q2?4::2p2?5->2qter), der(10) (10pter->10q24::18q2?3->18qter), der(18)(2pter->2p2?5::18p11.?2->18q?::18p11.?3->18pter) | del 18q21.1 (29 oligos) | 0.24, 51.19-51.34 | *De novo* | Yes | 250k SNP | FISH |
| 51 [28] | der(6)t(6;9)(p21.3;q22),ins(6;13)(p21.3;q?21q31), der(9)t(6;9), der(13)ins(6;13) | del 13q21.33-q21.2 (68 BACs)<br>del 13q22.3 (13 BACs)<br>See also Table 3.1.4B | 6.2, 69.5-75.7<br>1.1, 76.8-77.9 | *De novo*<br>*De novo* | Yes<br>Yes | 32k BAC | FISH and MLPA |

| Pt | Karyotype | Imbalance | Size (Mb)<br>Position (Mb) | *De novo*/<br>inherited | Clinical relevance[a] | Array platform | Confirmation method |
|---|---|---|---|---|---|---|---|
| A | At breakpoint region | | | | | | |
| 53 | der(2)t(2;7)(p21;q22),der(3) (7pter->7p13::3p26.3-> 3q21.3::11p13->11pter),der(7)(2pter->2p23.3::7p13->7q22::3q21.3->3qter),der(11)(3pter->3p26.3::2p12.2-> 2p23.3::11p13->11qter) | del 3q13.11q13.13 (53 BACs)<br>del 11p15.1 (3 BACs) | 2.8, 106.8-109.6<br>0.1, 21.3-21.4 | *De novo*<br>Unknown | Yes<br>? | 32k BAC | MLPA |
| B | Elsewhere in the genome | | | | | | |
| 10 | t(1;17)(p36.1;q11) | dup 1p34 (65 oligos) | 0.65, 45.67-46.32 | Unknown | ? | 244k Agilent | MLPA |
| 16 | t(2;10)(q22;q22.3) | del 5p13.1 (23 oligos) | 0.27, 38.54-38.81 | Unknown | ? | 244k Agilent | Illumina |
| 18 [25] | t(2;14)(q37.3;q13) | dup 1p34.1p33 (10 oligos) | 0.65, 45.86-46.51 | *De novo* | Yes [25] | 244k Agilent | FISH |
| 29 | t(5;10)(q33;q25) | del 2q33.3q34 (783 oligos)<br>del 9q21.13q21.2 (32 oligos)<br>del 12p11.22 (104 oligos) | 4.90, 207.86-212.76<br>0.20, 78.27-78.47<br>0.26, 29.98-30.24 | *De novo*<br>*De novo*<br>Paternal | Yes<br>?<br>No | 500k SNP | FISH |
| 30 | t(5;17)(p15.3;q25.3) | del 16p13.11 (10 BACs) | 0.9, 15.4-16.3 | Maternal | Yes [31,32] | 32k BAC | MLPA & FISH |
| 32 | t(6;9)(q21;p24) | dup 1q23.3 (34 SNPs) | 0.24, 160.75-160.99 | Maternal | No [29] | 250k SNP | MLPA |
| 51 | der(6)t(6;9)(p21.3;q22),ins(6;13)(p21.3;q?21q31),<br>der(9)t(6;9), der(13)ins(6;13) | del 1q21.1 (39 BACs)<br>See also Table 3.1.4A | 1.5, 144.7-146.3 | Paternal | Yes [33] | 32k BAC | FISH and MLPA |

[a] See Discussion.
[b] Because of the size of the deletion, no confirmation was performed.

single loss, two patients (6 and 53) had losses at multiple breakpoints and two patients (49 and 51) had multiple, non-overlapping losses in one breakpoint region. Patient 51 also had a loss elsewhere in the genome (Table 3.1.4B). In patient 6, with a loss at both breakpoints (1p22.1 and 6q15), the 1.1-Mb loss of chromosome 1 appeared to contain no known genes. The 4.25-Mb deletion in patient 42 contained the *FBN1* gene, explaining the observed Marfan phenotype. [22]

Five out of forty-six (11%) patients with a two-breakpoint chromosome rearrangement had a cryptic imbalance related to their reciprocal translocation. No imbalances were found related to inversions (*n*=6). Six out of eight (75%) patients with a CCR (more than two breakpoints) appeared to have an abnormal genome profile upon array analysis. All of these six patients had losses at the breakpoint regions.

## Imbalances elsewhere in the genome

Copy number changes elsewhere in the genome were present in seven patients: six patients had a reciprocal translocation and one patient had a CCR (Table 3.1.4B). Six losses and three gains not related to the breakpoints were detected in total in these seven patients. Only the patient with a CCR (51) had additional copy number alterations at one of the breakpoint regions (Table 3.1.4A). In this and two other patients (30 and 32), the respective CNV was inherited from a healthy parent. Patient 29 had three imbalances: two losses were *de novo* (on the paternal allele) and one loss was also observed in his healthy father.

In an adult patient (10) with a translocation (1;17)(p36.1;q11), a 650-kb gain in 1p34.1 was found. Unfortunately, this patient's parents were not available for further investigation. A partially overlapping, *de novo* 650 kb gain was found in a clinically more severely affected boy (18). Both gains overlap a 450-kb region in 1p34.1.

The last imbalance detected elsewhere was a 270-kb deletion at 5p13.1 in a patient with a translocation (2;10) (patient 16). Unfortunately, this patient's parents were not available for further investigation.

## Clinical criteria

All but three patients (*n*=51) could be scored according to the adapted clinical De Vries criteria (Tables 3.1.2 and 3.1.3). [17] Patient 43 died 1 day post-partum, patient 12 died at the

age of 2 months, and patient 52 had Sotos syndrome due to an *NSD1* mutation interfering with the phenotype. The distribution of the scores is shown in Supplementary Figure S3.1.1.

All patients with a chromosome imbalance in the breakpoint region (*n*=11) had a score of at least 3 with a median score of 5 (range 3-9). The highest score was found in the patient with imbalances both at a breakpoint and elsewhere (score 9 in patient 51). Patients with a chromosome imbalance restricted to elsewhere in the genome (*n*=6) tended to have a lower score (median 4.5, range 1-6). One of the imbalances in this group was considered not clinically relevant (see Discussion and Table 3.1.4B). Correction for this patient 32 led to a median score of 5 (range 1-6).



The median score in the total group with a possibly clinically relevant CNV (*n*=16) was 5 (range 1-9), while the median score in the group without a significant CNV (*n*=35) was 3 (range 0-7). The difference between these two groups is significant (*P*=0.002, Mann–Whitney *U*-test).

## DISCUSSION

In this study, 54 patients with an apparently balanced, *de novo* chromosome rearrangement were examined by high-resolution genome-wide array analysis. The mean number of CNVs, including well-known recurrent copy number polymorphisms, that was detected was 5.6 per patient (range 2-12). In general, the number of CNVs detected per patient depends on the platform and detection thresholds used, but the number found in our study does not differ substantially from patients without apparently balanced rearrangements. [18,29]

Out of 46 patients with a two-breakpoint chromosome rearrangement, 11 (25%) appeared to have an abnormal genome profile encompassing six losses, each at one of the breakpoints in five patients, and five losses and three gains elsewhere in the genome in six patients.

From analysis of their parents, two of the latter category could be specified as rare, inherited CNVs. Six out of eight patients with a CCR were found to have one or more clinically significant losses at one of the breakpoints. In addition, one of these six patients had a paternally inherited imbalance elsewhere in the genome. Although the overall percentage of patients with a cryptic or submicroscopic, clinically significant imbalance in this cohort is 31%, there is a remarkable difference between patients with a two-breakpoint chromosome rearrangement (24%) and those with a more complex rearrangement (75%).

The number of imbalances seen in our patient cohort is similar to the studies of Sismani *et al*. [11] and Baptista *et al*., [12] but lower than the studies of others (Table 3.1.1). [9,10,13-15] This might be due to differences in patient selection (reflected in the high number of aberrations found elsewhere in the genome in the studies of Gribble, Higgins and Gijsbers) and to the higher number of complex rearrangements studied by De Gregori. Compiling the data of the previous and present studies, we conclude that in almost half of the patients with a *de novo* chromosome rearrangement, a genomic imbalance can be detected by genome-wide array analysis. We confirmed that, in complex rearrangements, the chance of finding copy number alterations at the breakpoints is very high: 75 and 72%, in our study and the combined studies, respectively.

## Imbalances are not always located at breakpoints

In most patients (20%) with clinically relevant copy number alterations, the imbalance is detected in or near the breakpoints of the chromosomes involved (Table 3.1.4A). However, in 13% an imbalance is found elsewhere in the genome (Table 3.1.4B). As shown here and in previous studies, this was especially true for two-breakpoint *de novo* aberrations. In 19% of all patients with a two-breakpoint rearrangement, imbalances are found elsewhere. Especially in these cases, the clinical significance of the detected CNVs should be determined by parental analysis, among other investigations. The observed percentage of 19% is in agreement with the general figure of 17% of imbalances that is found in the DD/MCA population. [7,8] These results underline the importance of a genome-wide approach in patients with an apparently balanced, *de novo* chromosome rearrangement. If imbalances are found independent of the rearrangement breakpoints, this may have implications for the recurrence risk and warrants studies in the parents to exclude cryptic balanced translocations and insertions.

Furthermore, it is crucial to critically examine an apparently balanced rearrangement after initial detection, because they are often more complex than they appear at first.

## Losses are more frequent than gains at breakpoints

The clinically significant imbalances at the breakpoint regions found in this study were all deletions. Breakpoint deletions are more frequent in patients with a CCR than in patients with a two-breakpoint rearrangement. In the present study, we detected deletions in six out of eight CCR patients (75%). This is comparable to the results of De Gregori *et al.* [9] and Schluth-Bolard

*et al*., [14] who detected deletions in 69 and 80% of patients with a *de novo* CCR, respectively. Thus, deletions may be the main cause of phenotypic abnormalities in patients with a CCR.

The preponderance of deletions is similar to the results of others (Table 3.1.1). [9,10,12-14] Recently, Howarth *et al*. [30] showed that in breast cancer cell lines reciprocal translocations arising during mitosis may result in both deletions (up to 31 Mb) and duplications (up to 200 kb) at the breakpoint regions. They demonstrated that the underlying mechanism most likely is stalled replication bubbles during the interchromosomal exchange. *De novo* constitutional translocations have their origin during meiosis. Nonetheless, the same mechanism may cause imbalances during meiotic interchromosomal exchanges.



That we and others did not find breakpoint duplications in DD/MCA patients might be explained by their size (often under the detection threshold) and the fact that small duplications rarely result in a phenotype.

## Clinical significance of the detected imbalances

The size of the deletions and gains in our patients ranged from 100 kb to 15.3 Mb and from 240 to 650 kb, respectively. In patient 6 with deletions at both breakpoints, the abnormal phenotype was considered to be a consequence of the 9.2-Mb deletion at chromosome 6, because the small deletion at chromosome 1 did not contain any known genes. All other breakpoint deletions were considered pathogenic based on the criteria mentioned in Methods.

In four of the seven patients with an imbalance elsewhere in the genome, the imbalance was found to be inherited from a clinically unaffected parent. The deletion 16p13.11 (patient 30) and deletion 1q21.1 (patient 51) are known microdeletion syndromes with variable phenotypes. [31-33] Patient 51 also carries two significant losses at a breakpoint region, but we cannot exclude that the 1q21.1 deletion also contributes to the phenotype. The maternally inherited gain in 1q23.3 (patient 32) was considered unlikely to be clinically relevant because a larger gain has been detected in two control individuals from one study in the Database of Genomic Variants. [29]

The paternally inherited loss in patient 29 is in a gene-less region of 12p11.22 and therefore likely to be benign. Of the two *de novo* losses in the same patient (29), the 4.9-Mb loss in 2q33.3q34 is most likely to be clinically relevant. The 9q21.12q21.1 loss has not been detected before; and thus, its clinical significance remains uncertain, although a contribution to the clinical phenotype of patient 29 cannot be excluded.

The 650-kb gain in 1p34 in patient 10 is not a known polymorphism according to the Database of Genomic Variants, and is only partially overlapping gains that have been found in normal individuals (Nijmegen and Groningen in-house control data). Patient 18 had a similarly sized duplication, of which 450 kb overlapped with the gain of patient 10. The distal 200 kb, non-overlapping region, contains several genes, including *POMGNT1*. The phenotype of patient 18 is similar to previously published patients with larger overlapping duplications that included this gene. [25]

The 270-kb loss in 5p13.1 (patient 16) is not a known polymorphism but only contains the *LIFR* gene involved in autosomal recessive Stuve-Wiedemann syndrome, although the patient's clinical features do not resemble this syndrome. Unfortunately, the parents were unavailable for further studies and the clinical significance of the deletion remains unclear, as no similar microdeletion has been found in controls or other patients so far.

Thus, in at least four of the seven patients with imbalances elsewhere, the detected imbalance was considered to contribute to the abnormal phenotype.

## Clinical features pointing to an imbalance

All 16 patients with a potentially clinically relevant CNV showed DD, ranging from mild psychomotor or speech delay (in five patients) to severe DD (in seven patients). As discussed above, the gain in patient 32 with severe DD was, in retrospect, considered very unlikely to be causative for the phenotype. If we had only analyzed patients with an adapted De Vries score >3, we would not have missed any clinically relevant chromosome imbalances at the breakpoint regions (Supplementary Figure S3.1.1). This is in line with the results of the original study using De Vries criteria: all patients with a subtelomeric aberration had a De Vries score of at least 3. [17]

Two out of six patients with an aberration elsewhere in the genome had a score <3. This concerned the maternally inherited 1q23 gain in patient 32 (score 2) that was considered unlikely to be clinically relevant, and one 1p34 gain in patient 10 of uncertain clinical relevance (score 1). The median De Vries score of all 14 patients with a certainly clinically relevant CNV (Table 3.1.4) was 5 (range 3-9), while in the 35 patients without a relevant CNV the median score was 3 (range 0-7). Three patients could not be scored (see Results), and two patients had an imbalance of uncertain clinical relevance.

## Other mechanisms causing DD/MCA in balanced rearrangements

A truly balanced, *de novo* chromosome rearrangement may still contribute to an abnormal clinical phenotype due to disruption of a gene or due to a position effect. An example of the former was seen in patient 45 who appeared to have a disruption of the *TCF4* gene at 18q21.1, as described in a previous study. [27] Conventional methods for mapping chromosome breakpoints, such as FISH, are laborious, and often fail to identify the disrupted gene. Combining DNA array hybridization with chromosome sorting improves the efficiency of breakpoint mapping, but can only be applied when the physical properties of the derivative chromosomes allow them to be flow sorted. Nowadays more efficient and accurate breakpoint identification can be performed by next-generation paired-end sequencing. [34]



A position effect was most likely responsible for the split-hand-feet syndrome (SHFM) in patient 37 with an inversion breakpoint in 7q near the SHFM1 locus and the candidate genes *DSS1*, *DLX5* and *DLX6*. [26]

## Conclusion

The combined results of our study and previous reports show that in 79/171 (46%) of DD/MCA patients with a *de novo* chromosome rearrangement, a genomic imbalance could be detected by genomewide array analysis. In patients with a rearrangement involving more than two breakpoints, there is a high chance of detecting an imbalance at one of the breakpoints (21/29; 72%). In two-breakpoint rearrangements, an imbalance located at a breakpoint was detected in 26/142 (18%) patients. However, a substantial number of imbalances were also detected outside the breakpoint regions: in 33/171 (19%) patients, an imbalance was found elsewhere in the genome, which is comparable to the general DD/MCA population. In conclusion, diagnostic studies should not only focus on the rearrangement breakpoints, but a genome-wide approach should be used to investigate patients with apparently balanced, *de novo* chromosome rearrangements.

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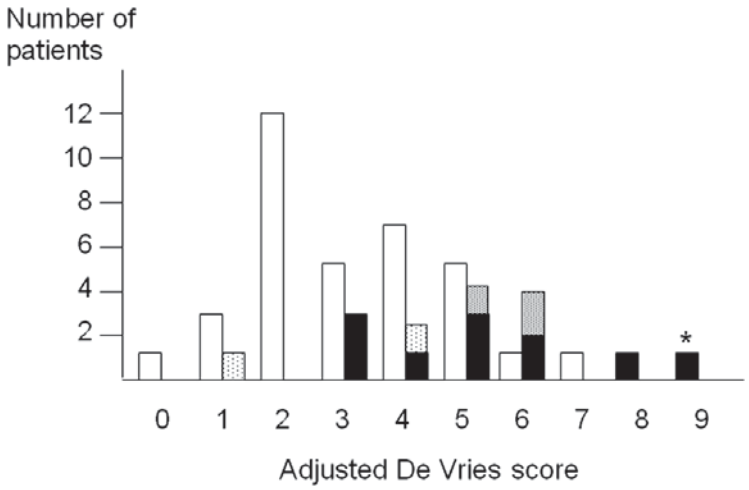
## Web resources

The URLs for data presented here are as follows:
Database of Genomic Variants (DGV), http://projects.tcag.ca/variation/
DECIPHER database, http://decipher.sanger.ac.uk/
European Cytogeneticists Association Register for Unbalanced Chromosome Aberrations (ECARUCA), http://ecaruca.net
Ensembl Human Genome Browser, http://www.ensembl.org/Homo_sapiens/
Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim
University of California-Santa Cruz Human Genome Browser, http://genome.ucsc.edu/




**Supplementary Figure S3.1.1** Distribution of the adjusted De Vries score (Table 3.1.2) in patients without clinically relevant imbalance ( ), patients with a clinically relevant imbalance at the breakpoint region ( ), patients with a clinically relevant imbalance elsewhere in the genome ( ), and patients with a potentially clinically relevant imbalance elsewhere in the genome ( ).
* Patient 51 who had imbalances at a breakpoint region and elsewhere in the genome.

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chapter 3.2

# Non-targeted whole genome 250K SNP array analysis as replacement for karyotyping in fetuses with structural ultrasound anomalies: evaluation of a one-year experience

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## ABSTRACT

We evaluated both clinical and laboratory aspects of our new strategy offering quantitative fluorescence (QF)-PCR followed by non-targeted whole genome 250K single-nucleotide polymorphism array analysis instead of routine karyotyping for prenatal diagnosis of fetuses with structural anomalies.

Upon the detection of structural fetal anomalies, parents were offered a choice between QF-PCR and 250K single-nucleotide polymorphism array analysis (QF/array) or QF-PCR and routine karyotyping (QF/karyo).

Two hundred twenty fetal samples were included. In 153/220 cases (70%), QF/array analysis was requested. In 35/153 (23%), an abnormal QF-PCR result was found. The remaining samples were analyzed by array, which revealed clinically relevant aberrations, including two known microdeletions, in 5/118 cases. Inherited copy number variants were detected in 11/118 fetuses, copy number variants with uncertain clinical relevance in 3/118 and homozygous stretches in 2/118. In 67/220 (30%) fetuses, QF/karyo was requested: 23/67 (34%) were abnormal with QF-PCR, and in 3/67, an abnormal karyotype was found.

Even though QF/array does not reveal a high percentage of sub-microscopic aberrations in fetuses with unselected structural anomalies, it is preferred over QF/karyo, as it provides a whole genome scan at high resolution, without additional tests needed and with a low chance on findings not related to the ultrasound anomalies.

## INTRODUCTION

Chromosome abnormalities have been reported in 9% to 27% of pregnancies in which (major) ultrasound (US) anomalies have been observed. [1-3] Traditional karyotyping, with a resolution of 5 to 10 Mb, is still the gold standard in prenatal cytogenetics. Array analysis, however, allows for a much higher resolution genome-wide scan of unbalanced genomic aberrations, but its application in prenatal diagnosis is still under debate, in particular because of the possible detection of copy number variants (CNVs) for which the clinical consequences are uncertain or unknown. Furthermore, there is a possibility of detecting CNVs in genomic regions that are related to late-onset diseases, such as deletions in regions including the *BRCA1* or *BRCA2* genes, or detecting a carrier status such as an intragenic deletion in the Duchenne muscular dystrophy gene. [4-7]



Targeted or low-resolution arrays were used in a number of laboratories. [8-15] These offer the advantage of being able to carefully select the genomic regions under study, with the ability to focus on regions with known clinical relevance. However, as we [16] and others [17] stated, the disadvantage of using a targeted approach is that currently unknown, clinically important aberrations might be missed, and targeted array platforms require continuous redesigning with the discovery of new disease loci. Furthermore, Coppinger *et al.* [18] showed that targeted with genome-wide backbone coverage array comparative genomic hybridization (aCGH) analysis reveals clinically significant submicroscopic abnormalities without an increase in unclear results or benign CNVs as compared with targeted aCGH.

The studies published so far on the use of array analysis in prenatal diagnosis all report on array analysis after karyotyping. [8-15,19-22] In our department of Human Genetics in Nijmegen, the Netherlands, the Affymetrix 250K genomewide single-nucleotide polymorphism (SNP) array has replaced routine chromosome studies since January 2009 as the first tier postnatal diagnostic test for patients with intellectual disability and/or congenital anomalies. Since October 2010, the same strategy is routinely followed in prenatal diagnosis if US structural anomalies are detected.

After extensive counseling, parents are offered a choice between routine karyotyping and non-targeted whole genome array analysis. If array analysis is preferred, a written informed consent needs to be signed by both parents, including the decision whether or not they want to receive information on aberrations unrelated to the US anomalies but possibly related to (late-onset) diseases.

The present paper reviews our experiences with this new strategy, including an evaluation of parental choices and the results of genetic analysis.

## MATERIALS AND METHODS

### Patients and samples

Since October 2010, pregnant women with US structural fetal anomalies (including nuchal translucency >3.5 mm), opting for an invasive procedure, were offered a choice between QF-PCR and 250K SNP array analysis or quantitative fluorescence (QF)-PCR and karyotyping. If the detected anomalies were not structural fetal anomalies (e.g., isolated poly-hydramnios or intrauterine growth restriction without other anomalies), if only so-called soft markers were seen (e.g., absent nasal bone, single umbilical artery or choroid plexus cysts), or if intrauterine fetal death was detected, women were excluded from the "choice" strategy.

Pretest and posttest counseling was carried out by the obstetrician–gynecologist, specifically trained for this by a clinical geneticist, through (individual) oral presentations and written information considering SNP array analysis.

Pretest counseling included oral and written information on the following:

- the resolution and detection rate (both strategies);
- the chance of detecting aberrations with unknown or uncertain clinical relevance (both strategies);
- the (low) possibility to detect an aberration, unlikely causative for the US anomalies, but with possibly clinical consequences for either the fetus or the parents themselves, including (lateonset) diseases for which screening and/or treatment is currently (un)available (array analysis strategy); and
- the necessity of collecting parental blood samples (array analysis strategy).

The clinical geneticist was always involved in posttest counseling if an aberration was detected by either strategy.

## Strategies

Regardless of the parental choice, all samples were first analysed by QF-PCR to test for one of the common aneuploidies of chromosomes 13, 18, 21, X, or Y. Samples with abnormal QF-PCR results were subsequently karyotyped to study whether the abnormality could be due to a parental rearrangement. After a normal QF-PCR result, either 250K SNP array analysis (QF/array strategy) or karyotyping (QF/karyo strategy) was performed, depending on the parental choice (Figure 3.2.1A).

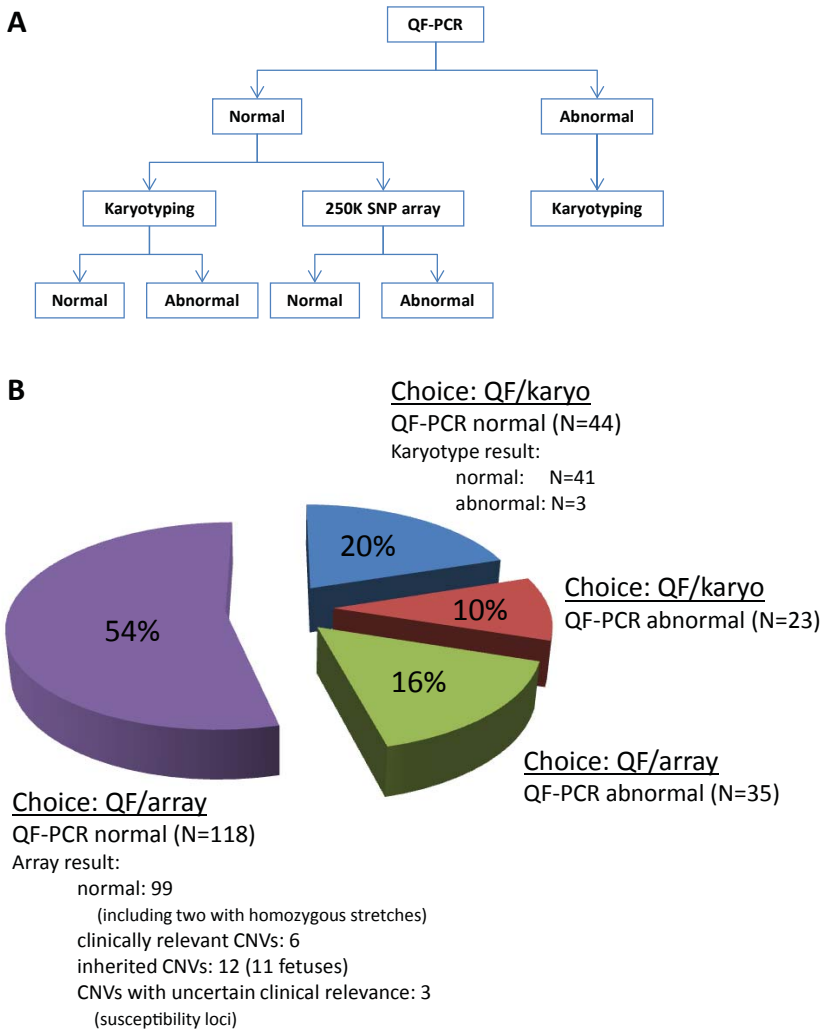


**Figure 3.2.1** Schematic overview of the new strategy **(A)** and the results in the QF/array and QF/karyo groups **(B)**.

For array analysis, informed consent from both parents was obtained, in which they signed for having received and understood sufficient information on the test and chose whether they wanted to be informed about CNVs unrelated to fetal US anomalies, but possibly related to (late-onset) diseases, subdivided in currently treatable or untreatable disorders.

Parental DNA was almost always available and simultaneously collected with the fetal sample.

## DNA isolation for array analysis

In case of chorionic villi, DNA was isolated from the mesenchymal core cell fraction of uncultured chorionic villi. For amniotic fluid, DNA was isolated from uncultured amniotic fluid cells in case of sampling at or after 20 weeks of gestation. Amniotic fluids sampled before 20 weeks of gestation were first cultured before DNA isolation was carried out to obtain sufficient DNA. DNA was isolated using the QIAamp MinElute Virus Spin Kit for DNA from uncultured amniotic fluid samples (following the "Purification of viral nucleic acids from plasma or serum" protocol) and the QIAamp DNA Mini Kit for cells from cultured amniotic fluid samples and chorionic villi (following the "DNA purification from blood or body fluids" spin protocol) (QIAgen, Westburg bv, the Netherlands). DNA concentrations used as input for array analysis were between 17 and 50 ng/L.

DNA from uncultured blood cells from parental blood was isolated following standard procedures (Chemagic Magnetic Separation Module 1 from Chemagen, Waltham, USA).

## QF-PCR

Fetal DNA was extracted using a Chelex-based procedure (Instagene Matrix, Bio-Rad Laboratories, CA). QF-PCR analysis was performed using the AneufastTM kit (version 1 or 2, Genomed Ltd, Kent, UK). For both the DNA extraction and QF-PCR analysis, instructions of the AneufastTM kit manufacturer were followed.

## Karyotyping

Routine cytogenetic analysis was carried out according to standard procedures.

## Affymetrix NspI SNP array hybridization and analysis

All array analyses were carried out using the Affymetrix GeneChip 250K (NspI) SNP array platform (Affymetrix, Inc., Santa Clara, CA, USA). Hybridizations, analysis, and data interpretation were performed according to the manufacturer's protocols and as described previously. [16,23] On the basis of the data published by Hehir-Kwa *et al*., [24] we set our 250K SNP array detection criteria for constitutional genome diagnostic applications at ≥150 kb for losses and ≥200 kb for gains ($p$-value >0.9 at a power of 95%). For carrier testing, these settings were the same for the region that was found aberrant in the index patient but was arbitrarily set at a threefold lower resolution for the remainder of the genome. Parents are considered healthy individuals unless otherwise indicated, and because their array data can be used as a control data set, we opted for this approach. The breakpoint positions of each aberrant region were converted to UCSC hg19 (UCSC Genome Browser, release February 2009) using hgLiftOver (http://genome.ucsc.edu/cgi-bin/hgLiftOver).



In addition to copy number analysis, SNP array data analysis also provided genotyping data and therefore, the possibility to detect homozygous stretches. This not only enabled the detection of uniparental disomy but might also reveal a possible candidate recessive disease gene in a homozygous stretch. [23]

Array analysis was not carried out in duplicate. To avoid sample mix-up as much as possible, all Qiagen-isolated DNA samples were additionally tested by QF-PCR prior to array analysis, to compare profiles and thus ensure that samples from the same pregnant woman were used for initial QF-PCR testing and subsequent array analysis. Furthermore, samples were loaded on the array platform with alternating sexes (known from the results of the QF-PCR), and in case parental samples were analyzed, patient–parent trio genotype analysis was routinely performed to rule out sample mix-up.

For efficiency reasons, array experiments were run twice a week. If array analysis was started after 20 weeks of gestation, fetal and parental samples were analyzed simultaneously to avoid a delay in reporting time. Because more time was available if array analysis was started before 20 weeks of pregnancy, parental DNA was only analyzed in case this was necessary for the interpretation of abnormal array results from fetal DNA.

## RESULTS

Between October 2010 and September 2011, a total of 220 samples (amniotic fluid or chorionic villi) from fetuses with structural US anomalies were sent to our laboratory for genetic analysis: In 70%, QF/array was chosen and in 30%, QF/karyo.

In the QF/array group, 23% of the samples showed an aberration with QF-PCR (16% of the total group); in the QF/karyo group, this was 34% (10% of total). Subsequent karyotyping showed all QF-PCR aberrations to be *de novo* aberrations, mostly so-called "free", non-inherited forms of trisomies 13, 18, or 21, monosomy X or triploidy, except for one with a 46, XY,der(14;21)(q10;q10)dn,+21 karyotype.

Figure 3.2.1B gives a schematic overview of these results, and in Table 3.2.1, an overview of the clinical features of the fetuses in both groups with normal QF-PCR results is given.

### QF/karyo strategy

Thirty percent of pregnant women (*n*=67) chose the QF/karyo strategy (Figure 3.2.1B). Of these, 66% (*n*=44) were normal with QF-PCR. In 3/44, subsequent karyotyping revealed

**Table 3.2.1** Overview of the clinical features of the fetuses in the QF/karyo and QF/array groups with normal QF-PCR results

| Ultrasound anomaly | QF/karyo group | QF/array group |
|---|---|---|
| *Multiple* | 7 [1] | 42 [2+1 [a]] |
| *Single* | | |
| Nuchal translucency / hygroma colli | 20 | 28 [1] |
| Congenital heart anomaly | 7 | 10 [1 [a]] |
| Brain anomaly | 2 [1] | 7 [1] |
| Cleft lip and palate | - | 2 |
| Hydrops / hydrothorax | 2 | 5 |
| Diaphragmatic hernia | 1 | 4 |
| Spina bifida | 2 | 1 |
| *Others* | 3 | 19 |
| TOTAL | 44 [2] | 118 [4+2 [a]] |

Between brackets: the number of fetuses in the specific group with an abnormal, clinically relevant karyotype or array profile.
[a] Not detectable with karyotyping.

an aberration. Details of the clinical features of these three foetuses and the aberrations are summarized in Table 3.2.2. Two of the three aberrations, 46, XX,r(15)(p?11.1q26.?2) and 46, XY,r(22)(p?11q?13), could be classified as "clinically relevant, explaining the US anomalies". Of the third aberration, 47, XY,+mar dn[3]/46,XY[26] in the long-term culture of chorionic villi only, this could not be established, as the marker was not further characterized and a cultural artifact could not be ruled out. However, considering the US anomaly of this fetus (anencephaly), the aberration is most likely not related to the US anomaly.

## QF/array strategy



Seventy percent of the couples (*n*=153) chose the QF/array strategy. Of these, 77% (*n*=118) were normal with QF-PCR and analyzed by array (Figure 3.2.1B). All array experiments resulted in data that met our quality criteria, and the mean reporting time of the results of QF-PCR and subsequent array analysis was 12.1 days (range 8-17 days) after sampling (if uncultured samples could be used and parental analysis was either carried out simultaneously or not needed).

In 62 cases (52%), parental array analysis was performed, either simultaneously with (*n*=60) or after fetal array analysis (*n*=2).

In 99/118 cases, a normal array result was obtained. In 6/118, a clinically relevant CNV was detected that could explain the US anomalies; in 11/118, one or two inherited, most likely benign CNVs were detected (in total 12 in 11 fetuses); and in 3/118, an inherited CNV was detected not explaining the US anomalies but involving a susceptibility locus for intellectual disability and autism (CNVs with uncertain clinical relevance).

One of these fetuses also had an inherited, not clinically relevant CNV. In two cases without CNVs, one or more homozygous stretches (>10 Mb) were detected. The parents of one of these two fetuses were known to be consanguineous and pretest counseled that there would be a higher percentage of common genetic material in the fetus.

There were no findings of CNVs related to (late-onset) diseases, without explaining the fetal US anomalies. Details of the US anomalies and the detected aberrations are summarized in Table 3.2.2. Of the six clinically relevant aberrations explaining the US anomalies, two were not cytogenetically visible: a 4.5-Mb interstitial loss in 4q21.1q21.21 (arr 4q21.1q21.21(77,514,977-82,004,241)x1 dn) and a 2.3-Mb loss in 22q11.21 (arr 22q11.21(18,895,227-21,277,471)x1 dn).

**Table 3.2.2** Overview of the clinical aspects of the fetuses in whom aberrations were detected, including pregnancy history, the fetal material that was used for DNA isolation, karyotyping and/or SNP array results, and the clinical relevance of the detected aberration

| Ultrasound anomalies | Gestational age at invasive procedure (weeks) | Pregnancy follow-up | Material used for DNA isolation | Karyotyping / SNP array result [a] | Clinically relevant? |
|---|---|---|---|---|---|
| **QF/karyo strategy** | | | | | |
| Diaphragmatic hernia, IUGR (P 2.3), congenital heart anomaly | 20.6 | TOP | UC AF | 46,XX,r(15)(p?11.1q26.?2).arr 15q26.2q26.3 (95,497,864-102,374,592 )x1 dn (6.9 Mb, 33 RefSeq genes) | Yes (explaining US) |
| Bilateral pyelectasy, white spot in the left ventricle of heart, choroid plexus | 20.2 | TOP | UC AF | 46,XY,r(22)(p?11q?13).arr 22q13.31 q13.33(45,189,299-51,175,626 )x1 dn (5.9 Mb, >50 RefSeq genes, among which *SHANK3*) | Yes (explaining US) |
| Anencephaly | 12.2 | TOP | - | 47,XY,+mar dn[3]/46,XY[26] (LTC) (cultural artifact cannot be excluded) | Unknown |
| **QF/array strategy** | | | | | |
| Spina bifida, large cyst (bladder) | 12.0 | TOP | UC CHOR | 46,XX,der(8)t(8;19)(p12;q13.33) dn.arr 8p23.3p12(190,568-37,367,826)x1, 19q13.3 3q13.43(49,745,289-59,039,699)x3 (8p23.3p12: 37.5 Mb, >50 RefSeq genes; 19q13.33q13.43: 9.3 Mb, >50 RefSeq genes) | Yes (explaining US) |
| NT 3.9 mm | 13.0 | TOP | C CHOR | 45,XX,der(14;18)(q10;10).arr 18p11.32 11.21(220,071-15,106,727)x1dn (14.8 Mb, >70 RefSeq genes) | Yes (explaining US) |
| Holoprosencephaly, renal agenesis, abnormal position of feet | 20.3 | TOP | UC AF | 46,XX,del(13)(q31.1).arr 13q31.1(87,099,840-87,502,737)x3 dn, 13q13. 2q34(88,033,666-115,074,878)x1 dn (13q31.1: 0.5 Mb, no RefSeq genes; 13q13.2q34: 27.3 Mb, >50 RefSeq genes) | Yes (explaining US) |

| Ultrasound anomalies | Gestational age at invasive procedure (weeks) | Pregnancy follow-up | Material used for DNA isolation | Karyotyping / SNP array result [a] | Clinically relevant? |
|---|---|---|---|---|---|
| Cystic hygroma, jugular sacs, congenital heart anomaly (AVSD), low-set ears, frontal bossing, abnormal profile | 16.5 | Ongoing pregnancy | C AF | 46,XX.arr 4q21.1q21.21(77,514,977-82,004,241)x1 dn (4.5 Mb, ~20 RefSeq genes) | Yes (explaining US) |
| Congenital heart anomaly (Fallot's tetralogy) | 21.5 | TOP | UC AF | arr 22q11.21(18,895,227-21,277,471)x1 dn (2.3 Mb, >50 RefSeq genes,) | Yes (explaining US) |
| Anencephaly (*No parental analysis yet: parents not simultaneously sampled, as TOP was planned because of US anomalies*) | 20.0 | TOP | UC AF | 46, XX,der(13)dup(13)(q32.1q14.11)del(13)(q32.1).arr 13q14.11q32.1(42,272,319-95,888,682)_3, 13q32.1q34(95,897,302-115,074,878)_1 (13q14.11q32.1: 53Mb; 13q31.1q34:19Mb. Both >50 RefSeq genes) | Yes (explaining US) |
| Spina bifida | 20.2 | TOP | UC AF | arr 22q13.31(46,847,579-47,492,189)x3 mat (645 kb; 4 RefSeq genes) | Most likely benign (inherited) |
| Renal agenesis, oligohydramnios, doligocephaly, cleft lip palate | 20.2 | TOP | UC CHOR | arr 11q22.1q22.3(100,330,608-103,683,448)x3 mat (3.3 Mb; 24 RefSeq genes) | Most likely benign (inherited) |
| IUGR and hyperechogenic bowel (*CF diagnostics was carried out: no CF*) | 21.3 | Pregnancy continued | UC AF | arr 2p16.3(51,294,688-51,490,709)x1 mat, 7q11.22(67,346,370-68,052,528)x3 mat (2p16.3: 196 kb, 7q11.22: 700 kb. Both: no RefSeq genes) | Most likely benign (inherited) |
| Congenital heart anomaly (AVSD), skeletal dysplasia (lethal) | 20.5 | TOP | UC AF | arr 3q28(190,692,992-191,189,965)x3 mat (500 kb; 4 RefSeq genes) | Most likely benign (inherited) |

*Table 3.2.2 continues on next page*

**Table 3.2.2** *Continued*

| Ultrasound anomalies | Gestational age at invasive procedure (weeks) | Pregnancy follow-up | Material used for DNA isolation | Karyotyping / SNP array result [a] | Clinically relevant? |
|---|---|---|---|---|---|
| NT 4.8 mm at 12.5 weeks (*At 20 weeks no structural US anomalies*) | 12.5 | Pregnancy continued | UC CHOR | arr Xq27.1q27.2(139,879,810-140,953,783)x3 pat (1 Mb; 10 RefSeq genes) | Most likely benign (inherited) |
| IUGR (<P5), oligohydramnios, SUA | 21.2 | Pre-eclampsia, child born at 27 weeks | UC AF | arr 7q36.2(153,342,469-153,608,121)x3 pat (265 kb; 1 RefSeq gene) | Most likely benign (inherited) |
| Spina bifida, ventriculomegaly | 20.4 | TOP | UC AF | arr 5q21.1(101,094,551-101,475,627)x1 pat (381 kb; no RefSeq genes) | Most likely benign (inherited) |
| Diaphragmatic hernia, dextrocardia | 20.4 | TOP | UC AF | arr 15q21.3(55,443,674-56,032,099)x3 pat (590 kb; 9 RefSeq genes) | Most likely benign (inherited) |
| Non-immune hydrops fetalis, polyhydramnios. | 29.3 | Ongoing pregnancy | UC AF | arr 10q25.3(116,155,718-116,571,796)x3 pat (416 kb; 2 RefSeq genes) | Most likely benign (inherited) |
| Ventriculomegaly | 20.4 | Ongoing pregnancy | UC AF | arr Xp22.2(11,456,334-11,948,913)x3 pat 492 kb, 2 RefSeq genes) | Most likely benign (inherited) |
| Anhydramnios, renal agenesis, congenital heart anomaly, spinal cord anomaly | 21.4 | TOP | UC CHOR | arr 10q21.1(55,502,542-55,908,282)x1 pat (400 kb; 1 RefSeq gene) 16p13.11(15,085,754-16,282,307)x3 pat (1.2 Mb; 15 RefSeq genes) | Most likely benign (inherited) Uncertain clinical relevance, not explaining US anomalies (susceptibility locus for autism and intellectual disability) |

| Ultrasound anomalies | Gestational age at invasive procedure (weeks) | Pregnancy follow-up | Material used for DNA isolation | Karyotyping / SNP array result [a] | Clinically relevant? |
|---|---|---|---|---|---|
| Anencephaly | 12.1 | TOP | UC CHOR | arr 1q21.1(145,479,219-147,814,694)x3 mat (2 Mb; >30 RefSeq genes) | Uncertain clinical relevance, not explaining US anomalies (susceptibility locus for autism and intellectual disability) |
| NT 5.5 mm | 12.2 | Pregnancy continued | UC CHOR | arr 1q21.1(146,101,297-146,953,752)x3 mat (600 kb; 4 RefSeq genes) | Uncertain clinical relevance, not explaining US anomalies (susceptibility locus for autism and intellectual disability) |
| IUGR (P5), enlarged arachnoidal space, small cerebellum, overlapping fingers, rocker bottom feet. | 21.3 | Child died postpartum | UC AF | Several homozygous stretches in the genome (Parents are consanguinous) | Potential, in case of unmasking recessive gene |
| Omphalocele, congenital heart anomaly, cleft lip and palate, abnormal hands, SUA, ventriculomegaly, hydrocephaly | 16.6 | Pregnancy continued, child died 1 day after birth | C AF | arr 8q13.3q21.3(73,279,485-92,600,075)x2 hmz (>50 RefSeq genes) | Potential, in case of unmasking recessive gene |

TOP, termination of pregnancy; UC, uncultured; C, cultured; AF, amniotic fluid; CHOR, chorionic villi; SNP, single-nucleotide polymorphism; QF, quantitative fluorescence; IUGR, intrauterine growth retardation; AVSD, atrioventricular septal defect; SUA, single umbilical artery; LTC, long-term culture.
[a] The second technique was only carried out for further characterization of a previously detected aberration.

Through the informed consent form, 46% of parents chose to receive information on CNVs unrelated to the fetal US anomalies, regardless whether or not a possibly associated (late-onset) disease was currently treatable. Thirty-three percent of couples chose not to be informed on such CNVs at all, and 21% chose to receive information only on CNVs related to diseases for which screening and/or treatment is currently available. In most cases, both parents chose the same options: only in two cases, parental choices differed.

## DISCUSSION

In our previous study, [16] we concluded that, despite potential results with unknown or uncertain clinical relevance, 250K SNP array analysis is ready for implementation in daily practice of prenatal diagnosis for pregnancies highly suspected for chromosomal aberrations. We now, indeed, offer non-targeted whole genome 250K SNP array analysis as replacement for karyotyping in the prenatal diagnostic setting of fetuses with structural US anomalies, a strategy that has been debated during the 15th meeting of the International Society for Prenatal Diagnosis in Amsterdam in July 2010. [25] In the present study, we have evaluated both laboratory and counselling aspects of this new strategy.

Seventy percent of the parents chose the QF/array strategy, a percentage biased by the fact that the obstetrician–gynecologists were not immediately familiar and confident with the new strategy. If this study had been based on the last 100 included pregnancies only, the percentage requesting QF/array would have been 82%. Assuming a nondirective counseling, these figures illustrate the parents' preferences for high-resolution analysis of the fetal material, even though more results with unknown or uncertain clinical relevance are expected, as compared with the lower resolution QF/karyo strategy. In the QF/karyo group, about one third of the foetuses showed an aberrant QF-PCR profile. A number of these foetuses were, on the basis of US findings, highly suspicious for an aberration detectable by QF-PCR, and therefore, the parents were not extensively counseled on the QF/array strategy before the results of the QF-PCR were known. In case of a normal result, parents could still opt for array analysis. This explains the higher percentage of abnormal QF-PCR results in the QF/karyo group compared with the QF/array group (34% vs. 23%). Considering the high overall percentage of aberrations detected with QF-PCR, the low costs, and the short turn-around time of this test, especially when performed each day in our laboratory, we prefer to perform QF-PCR first on all samples. With array run twice a week, this approach does not cause a delay in reporting time.

In the total group of 220 fetuses, 66 aberrations, causative for the US anomalies, were detected (30%): 58 by QF-PCR, two by karyotyping, and six by array analysis. Thus, in the group of 162 fetuses with normal QF-PCR results, 5% showed a clinically relevant aberration. Of the six of those detected by array analysis, two were only visible by array (1% of the 162 fetuses with normal QF-PCR results). The de novo 4.5-Mb loss in a fetus with multiple anomalies partially overlapped with a previously described microdeletion syndrome, caused by deletions in 4q21. [26] Except for the congenital heart anomaly, the phenotype of this fetus was comparable with the clinical features described by the group of Bonnet *et al*., and therefore, this 4q21 microdeletion was considered clinically relevant, explaining the US findings. The second submicroscopic aberration in the QF/array group was a *de novo* 2.3-Mb loss in 22q11.21, the known, recurrent 22q11 microdeletion, involved in the DiGeorge/VCF syndrome. [27] It was detected in a fetus with a heart anomaly suspected for this deletion and considered clinically relevant and causative for the US anomalies.



The other clinically relevant aberrations detected in the QF/array group are listed in Table 3.2.2 and all concerned large CNVs, both losses and gains that would also have been detected by routine karyotyping.

The 2% submicroscopic aberrations detected in the QF/array group (2/118) is in concordance with the percentages detected in the studies of Shaffer *et al*., [10] Kleeman *et al*., [15] and van den Veyver *et al*., [11] but it is much lower than the percentage we previously reported ourselves (16%). [16] In our previous study, only DNA samples of karyotypically normal fetuses, still highly suspected for a chromosomal aberration, were included. The inclusion criteria in the present strategy were less stringent and regardless of the karyotype. Therefore, one might opt for a list with US anomalies particularly suspect for a (sub)microscopic aberration. From the data obtained so far, however, such a list cannot be defined. Alternatively, as submicroscopic aberrations are often associated with intellectual disability, which cannot be diagnosed prenatally, one might also opt for even less stringent criteria for offering high-resolution array analysis.

Non-targeted whole genome SNP array analysis can not reveal only CNVs explaining the fetal US anomalies but also other types of CNVs, including inherited (benign) CNVs, CNVs of unknown or uncertain clinical significance (including CNVs located in regions containing so-called susceptibility loci), and CNVs related to (late-onset) diseases like hereditary breast cancer or Duchenne muscular dystrophy. In the present study, we detected inherited (benign) CNVs in 11 fetuses (8%), CNVs with uncertain clinical relevance (in regions with susceptibility

loci) in three fetuses (2%), and no CNVs related to (late-onset) diseases. In the QF/karyo group, however, we also detected one aberration with unknown clinical relevance (47, XY,+mar dn[3]/46, XY[26]; 1%), thereby underscoring the fact that the issue of obtaining results with unknown or uncertain clinical relevance is not new and only restricted to new technologies but is related to (genetic) testing in general. Even though more results causing counseling difficulties and stress for the pregnant couple are expected with the non-targeted whole genome approach as compared with karyotyping, our results do not confirm this expectation, and we did not find results that are impossible to interpret, as expected by de Jong *et al.* [7] Of the three pregnancies with CNVs with uncertain clinical relevance (susceptibility loci), two were terminated because of the US anomalies and the third pregnancy was continued. The carrier father of the fetus with the 16p13.11 gain had learning difficulties and epilepsy, features which are known to be possibly related to the 16p13.11 gain. [28] The carrier parent of the arr 1q21.1(146,101,297-146,953,752)x3 gain was not clinically evaluated. The carrier parent of the arr 1q21.1 (145,479,219-147,814,694)x3 gain was phenotypically normal, although her brother (not array analyzed yet) showed autistic features.

Rare inherited CNVs cannot always be classified as benign, but they are, depending on the size, gene content, and type of CNV (gain/loss), less likely to directly lead to a clinical phenotype. Small CNVs (<0.1 Mb) and gains are less likely to be pathogenic than large CNVs (>1 Mb) and losses, respectively. [29,30]

Moreover, inherited CNVs are usually not related to physical anomalies, yet rather to susceptibility for mental disorders, such as autism spectrum disorder. The inherited CNVs, we categorized as "most likely benign", were indeed all but one <1 Mb in size.

Copy number variants with unknown or uncertain clinical relevance and those involved in (late-onset) diseases raise ethical questions, particularly in prenatal diagnosis. We evaluated postnatal array data from more than 7500 samples from patients and parents and estimated the frequency of a CNV unrelated to the referral reason but possibly related to a (late-onset) disease to be <0.1% (unpublished results). In spite of this low frequency, in our prenatal strategy, we started counseling parents about the possibility to detect such a CNV and asked them whether they want to be informed on such a finding. The parental choices were diverse: Almost half of all parents wanted to be informed on CNVs related to both treatable and untreatable traits, 33% did not want to be informed at all on unrelated CNV findings, and 21% only wanted to be informed about CNVs related to diseases that can currently be screened or treated for. In routine practice, we did not find any such CNV in the group included in

this study. This is in line with the findings of Srebniak *et al.*, [22] who, in their study on array analysis in prenatal diagnosis, also did not find any fetal CNV with a possible adverse effect later on in life. In their pretest counseling policy, they included information about results not explaining the US anomalies but with a possible adverse health effect later on in life and made a distinction between infancy/childhood and adulthood. They also included a choice option to be informed about new information from future studies. We do agree with the group of Srebniak that adequate and sufficient information should be offered during counseling, but we believe that there should be an appropriate balance to prevent unnecessary anxiety in the future parents. Therefore, we decided to limit the number of choice options and not to mention in detail the possibility of uncovering nonpaternity, consanguinity, or incest (as reported by Schaaf *et al.* [31]). Even though up till now no cases of nonpaternity nor incest have been detected in our prenatal setting, one might opt for the development of international practice guidelines concerning issues of consent, result disclosure, and reporting of array results in both prenatal and postnatal settings.



To summarize, we have evaluated several aspects of our new strategy in routine prenatal diagnosis of replacing karyotyping by non-targeted, whole genome 250K SNP array analysis in fetuses with structural US anomalies. In our experience, most future parents prefer the high resolution QF/array strategy, even though this can lead to unsolicited findings. In the QF/array group, only two (2%) clinically relevant submicroscopic aberrations were detected. Despite this low frequency, which can be explained by the broad inclusion criteria, we do promote the use of non-targeted whole genome array analysis in this group of fetuses, as it provides a more accurate and reliable whole genome scan within the same time frame as karyotyping, and all clinically relevant aberrations detected in the QF/karyo group would have been detected with the QF/array strategy as well, but not vice versa. Furthermore, in our experience, the disadvantages of high-resolution array analysis, that is, detecting CNVs with unknown or uncertain clinical relevance or CNVs causative for (late-onset) diseases unrelated to the US anomalies, do not outweigh the advantages of the high resolution of array analysis. Concluding from our findings, during pretest counseling, the focus should not be on the finding of CNVs possibly related to (late-onset) diseases. However, as in 8% of the fetal samples, an inherited, most likely benign, CNV was detected, and in another 2%, a CNV with uncertain clinical relevance was found, parents should be well informed about such findings, and parental blood samples should always be available.

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chapter 4.1

# Genotype-phenotype mapping of chromosome 18q deletions by high-resolution array CGH: an update of the phenotypic map

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## ABSTRACT

Partial deletions of the long arm of chromosome 18 lead to variable phenotypes. Common clinical features include a characteristic face, short stature, congenital aural atresia, abnormalities of the feet and mental retardation. The presence or absence of these clinical features may depend on the size and position of the deleted region. In reverse, it is also known that patients whose breakpoints are localized within the same chromosome band may exhibit distinct phenotypes. New molecular techniques such as array CGH allow for a more precise determination of breakpoints in cytogenetic syndromes, thus leading to better-defined genotype-phenotype correlations. In order to update the phenotypic map for chromosome 18q deletions, we applied a tiling resolution chromosome 18 array to determine the exact breakpoints in 29 patients with such deletions. Subsequently, we linked the genotype to the patient's phenotype and integrated our results with those previously published.

Using this approach, we were able to refine the critical regions for microcephaly (18q21.33), short stature (18q12.1-q12.3, 18q21.1-q21.33 and 18q22.3-q23), white matter disorders and delayed myelination (18q22.3-q23), growth hormone insufficiency (18q22.3-q23) and congenital aural atresia (18q22.3).

Additionally, the overall level of mental retardation appeared to be mild in patients with deletions distal to 18q21.33 and severe in patients with deletions proximal to 18q21.31. The critical region for the 'typical' 18q- phenotype is a region of 4.3 Mb located within 18q22.3-q23. Molecular characterization of more patients will ultimately lead to a further delineation of the critical regions and thus to the identification of candidate genes for these specific traits.

## INTRODUCTION

Chromosome 18 abnormalities including deletion 18q, deletion 18p and ring (18) are among the most frequently occurring autosomal anomalies, together occurring in approximately 1/40,000 live births. [1] Of these abnormalities, deletions of the long arm of chromosome 18 are the most common, including the typical (distal) 18q- syndrome and proximal interstitial 18q deletions.

The typical 18q- syndrome, also named De Grouchy syndrome, is caused by distal chromosome 18q deletions and was first described in 1964. [2] Numerous reports have been published since. [1,3-7] The most common manifestations of patients with 18q- syndrome are short stature, microcephaly, midface hypoplasia, hypertelorism, congenital aural atresia, foot deformities, mental retardation and hypotonia. [1,3,7]



Compared to distal deletions, proximal interstitial 18q deletions involving bands q12 to q21 have been encountered less frequently. [8-11] Clinical features described in patients with proximal interstitial 18q deletions are prominent forehead, midface hypoplasia, strabismus, seizures and behavioural problems.

The combined literature suggests that a wide phenotypic spectrum exists among patients with 18q deletions, and that distinct genotype-phenotype correlations await to be defined.

Array-based comparative genomic hybridization (array CGH) has been proven to be effective in determining molecular profiles with a resolution 5-10 times higher than conventional karyotyping methods [12-14] and has shown to be useful in defining critical regions for phenotypic traits, including congenital aural atresia in De Grouchy syndrome. [15]

In this study, we analysed clinical and molecular data of 29 patients with cytogenetically visible 18q deletions in order to establish a genotype-phenotype map for 18q. We employed tiling resolution array CGH to determine the exact size and position of the chromosome aberrations. By comparing the extent of the deletions with the respective phenotypes, significant genotype-phenotype correlations were established.

## PATIENTS AND METHODS

### Patients

A series of 29 patients was selected for this study. All patients were examined by a clinical geneticist. G-banded karyotyping (650 band level) was performed and all patients were

diagnosed either with a cytogenetically visible deletion or with clinical features suggesting chromosome 18 abnormalities. Genomic DNA was isolated from peripheral blood lymphocytes of all patients, using a QIAamp kit according to the instructions of the manufacturer (QIAgen, Venlo). For the genomic array experiments, reference DNA was isolated from cytogenetically normal and healthy individuals using the same QIAamp kit (QIAgen, Venlo).

Patients 6, 8-15, 18-20, 22-24 and 26-27 were previously described and patient 28 was previously described. [15,16]

## Array CGH

The array contained 1424 chromosome 11 BAC clones and 815 chromosome 18 BAC clones, all part of the 32K fingerprint validated BAC clone set. [17] This resulted in a more than two times coverage of chromosome 18 with an average of 1 clone per 93 kb. All clones were spotted in sixfold onto UltraGAPS slides (Corning) using an Omnigrid 100 arrayer (Genomic Solutions, Ann Arbor). Array CGH was performed essentially as described before, with minor modifications. [14,18] In brief, the chromosome 18 array CGH profiles were established through co-hybridization of 500 ng Cy3-dUTP and Cy5-dUTP (Amersham Biosciences, Buckinghamshire) labeled test and reference DNA, using a GeneTac Hybridization station (Genomic Solutions, Ann Arbor). After scanning, test-over-reference ratios (T/R values) were determined for each clone and log2-transformed. Log2 T/R values were normalized per array subgrid on the basis of the average logarithmic fluorescent intensities of the chromosome 11 BAC clones by Lowess curve fitting with a smoothing factor of 0.1 to predict the log2-transformed test-over-reference (T/R) value. [19] Log2 T/R ratios for chromosome 18 BAC clones were calculated on the basis of the smoothing curve through the ratios of the chromosome 11 BAC clones. After data normalization, an automated statistical procedure based on a Hidden Markov Model (HMM) algorithm was used for detection of genomic copy number changes and precise localization of start- and end-points of each copy number variation.

### *Array validation*

To test the specificity and sensitivity of the tiling resolution chromosome 11/18 BAC array, we performed a series of validation experiments. To test the specificity of the array, two normal-versus-normal control hybridizations (including a dye-swap) using normal healthy blood donors were conducted. All clones showed log2 intensity ratios in between the *a priori* set thresholds for copy-number gain (>0.3) or loss (<-0.3). Additionally, the HMM

algorithm did not detect any abnormalities and marked all clones as normal, indicating that the array is specific. Next, we tested the sensitivity of the array by hybridizing DNA from two patients with known (sub) microscopic chromosome 18 abnormalities to the array. After optimization of the normalization procedure using the chromosome 11 BAC clones also present on the array, the two chromosome 18 abnormalities as detected in previous studies and by conventional karyotyping, were readily identified by the HMM algorithm using the tiling chromosome 18 array.

In conclusion, the sensitivity of the array for detection of (sub) microscopic deletions and duplications was validated and the specificity of the array was assured by performing a stringent normalization procedure.



***FISH validation experiments***

DNA from chromosome 18 BAC clones selected for FISH validation was amplified with the TempliPhi Large Construct DNA Amplification Kit (Amersham Biosciences) according to the instructions of the manufacturer. FISH validation experiments were performed on metaphase spreads prepared from patient-derived cell lines. Probe labeling, slide preparation and hybridization were carried out essentially as described before. [20]

## RESULTS

### Cytogenetic karyotypes

Upon routine karyotyping, 28 of the 29 patients showed 18q deletions. Six patients had a proximal interstitial deletion and 22 patients had a terminal deletion. Patient number 6 presented with a complex aberration affecting chromosomes 7q, 18p and 18q. In patients number 8 and 10, the distal deletion was present in a mosaic fashion (33% and 75%, respectively). Patients 16 and 18 had a ring chromosome 18 with deletions of only q-arm segments. Patient number 28 was previously diagnosed with Rasmussen syndrome, which is characterized by bilateral atresia of the external auditory canals, vertical talus and increased interocular distance. [16,21] She had a normal GTG-banded karyotype, but a submicroscopic 18q deletion was suspected due to the fact that she displayed several features of 18q- syndrome, i.e. congenital aural atresia, typical shape of the ears with prominent crus of the helix, hypertelorism and clubfeet. Patient number 29 had, in addition to the 18q deletion, an 18p duplication due to an unbalanced pericentric inversion.

**Table 4.1.1** Overview of the cytogenetic results of the 29 patients

| Patient number | Conventional karyotypes | Molecular karyotypes | Megabase positions | Refinement |
|---|---|---|---|---|
| 1 | 46,XY,del(18)(q11.1q21.1) | 46,XY,del(18)(q11.2q21.1) | 18.9 - 42.9 | + |
| 2 | 46,XY,del(18)(q21.2q22.1) | 46,XY,del(18)(q12.1q21.1) | 25.2 - 42.9 | + |
| 3 | 46,XX,del(18)(q21.1q21.3) | 46,XX,del(18)(q21.1q21.33) | 46.8 - 59.5 | + |
| 4 | 46,XY,del(18)(q21.3) | 46,XY,del(18)(q21.1q22.2) | 47.2 - 65.7 | + |
| 5 | 46,XY,del(18)(q21.1q21.3) | 46,XY,del(18)(q21.1q22.1) | 47.9 - 61.3 | + |
| 6[a] | 46,XY,der(7)t(7;18)(q36.1;q23)(18)(pter->q23::p11.2pter),del(18)(q22.3q23) | 46,XY,der(7)t(7;18)(q36.1;q23)(18)(pter->q23::q11.1pter),del(18)(q22.3q23) | 67.7 - 74.9 | + |
| 7 | 46,XY,del(18)(q21.2) | 46,XY,del(18)(q21.2) | 49.0 - 76.0 | - |
| 8 | 46,XY[67%]/46,XY,del(18)(q21.31)[33%] | 46,XY[67%]/46,XY,del(18)(q21.2)[33%] | 51.2 - 76.0 | + |
| 9 | 46,XY,del(18)(q21.2) | 46,XY,del(18)(q21.31) | 53.3 - 76.0 | + |
| 10 | 46,XY[25%]/46,XY,del(18)(21.3)[75%] | 46,XY[25%]/46,XY,del(18)(21.31)[75%] | 53.3 - 76.0 | + |
| 11 | 46,XX,del(18)(q21.31) | 46,XX,del(18)(q21.31) | 54.1 - 76.0 | - |
| 12 | 46,XX,del(18)(q21.31) | 46,XX,del(18)(q21.31) | 54.4 - 76.0 | - |
| 13 | 46,XY,del(18)(q21.31) | 46,XY,del(18)(q21.31) | 54.9 - 76.0 | - |
| 14 | 46,XY,del(18)(q21.32) | 46,XY,del(18)(q21.32) | 55.1 - 76.0 | - |
| 15 | 46,XX,del(18)(q21.3) | 46,XX,del(18)(q21.32) | 57.4 - 76.0 | + |
| 16 | 46,XY,r(18)(p?q21.31) | 46,XY,r(18)(q21.33) | 57.1 - 76.0 | + |
| 17 | 46,XY,del(18)(q21.3) | 46,XY,del(18)(q21.33) | 58.1 - 76.0 | + |
| 18 | 46,XX,r(18)(pterq23) | 46,XX,r(18)(q21.32) | 58.5 - 76.0 | + |
| 19 | 46,XY,del(18)(q21.3) | 46,XY,del(18)(q21.33) | 59.1 - 76.0 | + |
| 20 | 46,XY,del(18)(q22) | 46,XY,del(18)(q22.1) | 61.4 - 76.0 | + |
| 21 | 46,XX,del(18)(q22) | 46,XX,del(18)(q22.1) | 61.8 - 76.0 | + |
| 22 | 46,XX,del(18)(q21.33) | 46,XX,del(18)(q22.1) | 63.0 - 76.0 | + |
| 23 | 46,XX,del(18)(q22.3) | 46,XX,del(18)(q22.1) | 63.1 - 76.0 | + |
| 24 | 46,XX,del(18)(q22.1) | 46,XX,del(18)(q22.1) | 64.0 - 76.0 | - |
| 25 | 46,XX,del(18)(q22) | 46,XX,del(18)(q22.2) | 65.1 - 76.0 | + |
| 26[a] | 46,X,del(X)(q21.2),der(18)t(X;18)(q21.2;q22) | 46,X,del(X)(q21.2),der(18)t(X;18)(q21.2;q22.3) | 67.7 - 76.0 | + |
| 27 | 46,XY,del(18)(q22.2) | 46,XY,del(18)(q22.3) | 68.8 - 76.0 | + |
| 28 | 46,XX | 46,XX,del(18)(q22.3) | 69.1 - 76.0 | + |
| 29 | 46,XX,der(18)(pter->q23::p11.2->pter).ish del(18)(q23) | 46,XX,der(18)(pter->q23::q11.1->pter),del(18)(q22.3) | 69.9 - 76.0 | + |

[a] Breakpoints in present study in concordance with detailed FISH validation of previous study Veltman *et al*. (2003).

Karyotypes from all patients were re-evaluated and the cytogenetic diagnoses were confirmed (Table 4.1.1).

## Molecular karyotypes

A total of 29 patients was molecularly characterized by array CGH using the tiling resolution array. Of these, 18 patients were described in a previous study defining a critical region for CAA. [15]

In all 29 patients a deletion of chromosome 18q was detected. The array CGH approach resulted in a more precise delineation of the deleted region in 23 cases and the cytogenetic diagnoses could be refined (Table 4.1.1).



The six interstitial deletions ranged in size from 7.2 Mb to 24.0 Mb, with proximal breakpoints ranging from 18q11.2 (18.9 Mb) to 18q22.3 (67.7 Mb) and distal breakpoints ranging from 18q21.1 (42.9 Mb) to 18q23 (74.9 Mb), respectively. All six deletions were unique since none of the patients shared a common breakpoint (Figure 4.1.1a). A representative chromosome 18 profile of an interstitial deletion is shown in Figure 4.1.1b.

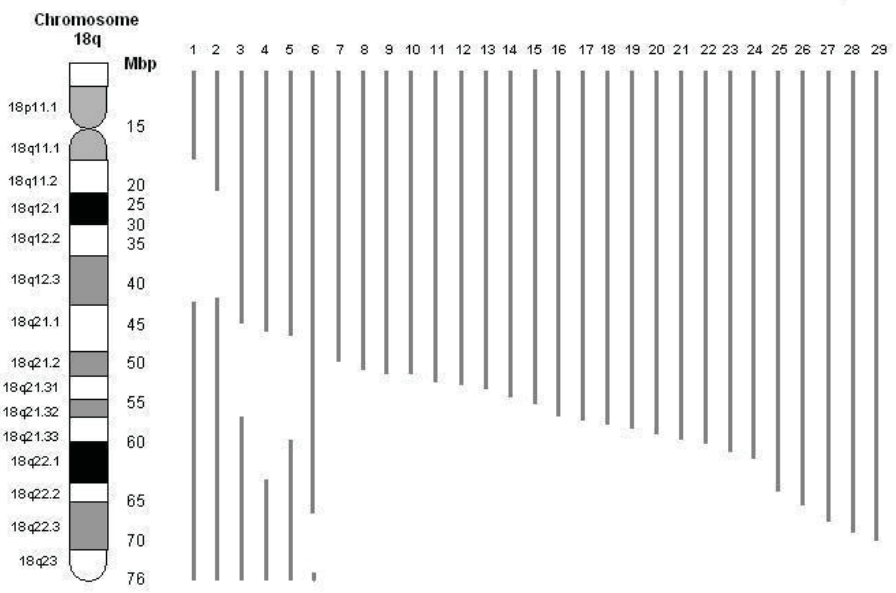


**Figure 4.1.1a** Overview of the array CGH results of the 29 patients. At the left the ideogram of chromosome 18 and the corresponding megabase positions (Mb) are shown. The chromosome material present in each patient is indicated by a grey line.

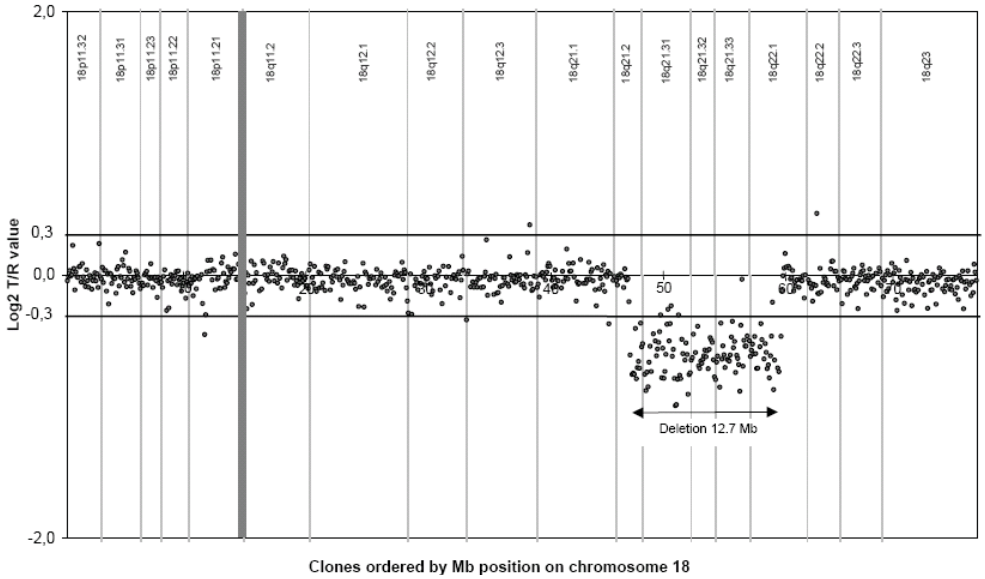


**Figure 4.1.1b** Chromosome 18 plot of Patient 3, as obtained by array CGH. BAC clones on the microarray are indicated by dots, representing the log2-transformed and normalized test over reference intensity ratios [log2(T/R)], ordered on Mb position from pter to qter. The 12.7 Mb deletion of 18q21.1q21.33 detected by HMM analysis is indicated by a double-sided arrow.

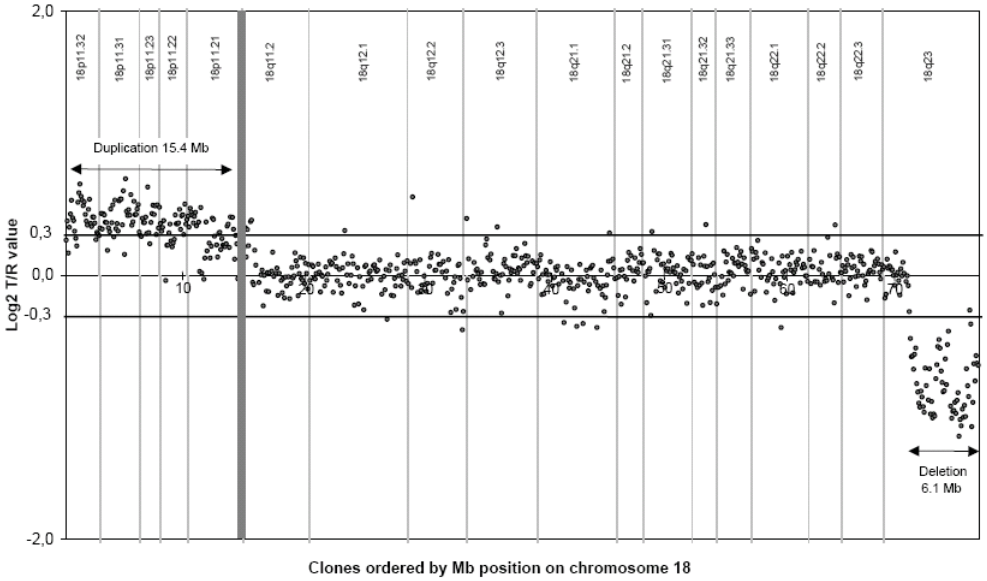


**Figure 4.1.1c** Chromosome 18 plot of Patient 29, as obtained by array CGH, showing a duplication of 18p11.1pter and a 6.1 Mb terminal deletion of 18q23qter.

The size of the 23 terminal deletions varied considerably, with the largest deletion spanning 27.0 Mb in size, whereas the smallest deletion encompassed 6.1 Mb of genomic sequence, thus the proximal breakpoints ranged from 18q21.2 to 18q22.3 (Figure 4.1.1a). This latter submicroscopic 6.1 Mb deletion was identified in patient number 29 who also showed a microscopically visible duplication on the short arm of chromosome 18 (Figure 4.1.1c). All 23 patients exhibited genuine terminal deletions since no distal interstitial deletions were detected by array CGH. An overview of all deletions detected in 18q including the location and size is shown in Figure 4.1.1a.

## Genotype-phenotype correlations



After high-resolution chromosome 18 profiles were obtained from all the patients included in this study, we made an attempt to correlate the clinical findings of the patients (Table 4.1.2) with the location and size of the deletions identified by array CGH (Table 4.1.1).

### *Microcephaly*

Microcephaly is defined as a reduction in head circumference greater than 2.5 SD below the mean value for age and sex. Head circumference was measured at different ages in all patients. Microcephaly was consistently detected in four patients with an interstitial deletion and in eight patients with a terminal deletion (patient numbers 3-6 and 11-18). The smallest overlapping region in this group of patients is a region of approximately 1 Mb, between 58.5 and 59.5 Mb, located in band 18q21.33.

### *Short stature*

The definition of short stature is a height of more than 2 SD below the mean value at a certain age, corrected for sex and race. This clinical feature is commonly described in patients with 18q deletions. [1,22] In all patients, height was determined multiple times. Only four of our 29 patients had a height measure of 0 SD or higher for their age. A total of 22 of 29 patients had a height below average and of these, 15 were officially reported with short stature (< -2 SD). These 15 patients showed three regions of deletion overlap, including the regions 18q12.1-q12.3 (25.2-42.9 Mb), 18q21.32-q21.33 (58.5-61.3 Mb) and 18q22.3-q23 (67.7-74.9 Mb).

In two patients with short stature, IGF1 was determined and provocative tests using arginine hydrochloride or clonidine were performed in order to measure growth hormone release. A growth hormone deficiency was detected in one patient (number 18).

**Table 4.1.2** Overview of the clinical features in 29 patients

| Patient number | Age | Micro-cephaly[a] | Stature | CAA | Cleft palate/ lip | Foot deformities[b] | Delayed myelination | Epilepsy | Level of MR | GH deficiency | Low IgA level |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 25 | - | < -2 SD | - | - | - | | - | severe | | - |
| 2 | 19 | - | < -2.5 SD | - | CP | - | | - | severe | | - |
| 3 | 19 | + | < -2.5 SD | - | - | - | | - | severe | | - |
| 4 | 26 | + | 0 SD | - | - | - | | - | severe | | - |
| 5 | 6 | + | - 2.5 SD | - | - | - | | - | severe | | |
| 6 | 14 | + | -2.5 SD | + | CP | + | | - | severe | | |
| 7 | 8 | - | - 2.5 SD | - | - | - | | + | severe | | + |
| 8 | 11 | - | 0 SD | + | - | - | - (age 5 yrs) | - | moderate | | - |
| 9 | 27 | - | - 2 SD | + | - | - | | + | moderate | | |
| 10 | 12 | - | - 2.2 SD | + | - | + | | + | moderate | | - |
| 11 | 14 | + | - 2-2.5 SD | + | - | + | | - | moderate | | |
| 12 | 9 | + | < - 2.5 SD | + | - | - | | - | mild | | - |
| 13 | 44 | + | < -2.5 SD | + | - | + | | + | severe | | |
| 14 | 20 | + | -1.5 SD | + | CL/CP | + | | - | mild | | - |
| 15 | 14 | + | -1.5 SD | + | - | - | | - | mild | | - |
| 16 | 39 | + | | | - | + | | - | moderate | | |

| Patient number | Age | Micro-cephaly[a] | Stature | CAA | Cleft palate/ lip | Foot deformities[b] | Delayed myelination | Epilepsy | Level of MR | GH deficiency | Low IgA level |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 13 | + | -1.8 SD | - | - | + | | - | severe | | + |
| 18 | 6 | + | < -2.5 SD | + | CP | + | | - | moderate | + | - |
| 19 | 10 | - | - 1.7 SD | + | CL/CP | - | + (age 5 yrs) | - | severe | | - |
| 20 | 17 | - | < -1.5 SD | + | - | - | | - | mild | - | - |
| 21 | 23 | - | | | - | - | | - | mild | | |
| 22 | 26 | - | < -1.5 SD | + | - | + | | - | mild | | - |
| 23 | 22 | - | - 2.2 SD | + | - | + | | - | mild | | - |
| 24 | 8 | - | - 2.5 SD | + | - | - | | - | normal | | |
| 25 | 9 | - | - 2.5 SD | + | - | + | | - | mild | | |
| 26 | 8 | - | - 1.5 SD | + | CP | - | - (age 5 yrs) | - | mild | | - |
| 27 | 44 | - | +1 SD | + | - | + | | - | mild | | |
| 28 | 11 | - | | + | - | + | | | normal | | |
| 29 | 5 | - | 0 SD | + | - | - | + (age 4 yrs) | - | mild | | |

CAA, congenital aural atresia; MR, mental retardation; GH, growth hormone; IgA, immunoglobulin A.
[a] Microcephaly is defined as a head circumference >2.5 SD below the mean for age and sex.
[b] Foot deformities include vertical talus, clubfoot, metatarsus adductus, pes cavus, and calcaneovalgus.

### *Congenital Aural Atresia*

Congenital aural atresia (CAA) may vary from a mild abnormality with narrowing of the external auditory canal and hypoplasia of the tympanic membrane and middle ear cavity to entire absence of the middle ear in combination with anotia, bony atresia and hypoplasia of inner ear structures. [23] CAA was seen by an otholaryngologist in all but one patient (patient number 7) with terminal deletions. In two patients (number 16 and 21), it is unknown. Patients with CAA shared a common deleted region of ~5 Mb located in 18q22.3-qter (69.9-74.9 Mb).

### *Cleft palate with or without cleft lip*

Cleft palate with or without cleft lip (CP/CL) is sometimes described in patients with 18q deletions. [1,24,25] In our series, four patients had a cleft palate and two patients had both a cleft palate and cleft lip. Of the six patients with CP/CL, two patients had a proximal interstitial deletion (patients number 2 and 6) and four patients had terminal deletions (patients number 14, 18, 19 and 26). These data indicate a proximal critical region located in 18q12.1-q12.3 (25.2-42.9 Mb) and a distal critical region located in 18q22.3-23 (67.7-74.9 Mb).

### *Mid- and forefoot deformities*

Abnormalities of the lower extremities are often encountered in patients with chromosome 18q aberrations. [1,3,7,26] A total of 13 patients in our study cohort had mid- and forefoot deformities, like clubfoot, vertical talus, pes planus or pes cavus. Patients number 1-5 with a proximal interstitial 18q deletion did not show any foot deformities. A common deleted region for abnormalities of the lower extremities was located in 18q22.3-q23 (69.1-74.9 Mb).

### *White matter alterations and delayed myelination*

White matter alterations and delayed myelination have been described in a number of patients with terminal 18q deletions. [27-30] In our cohort, four patients with a terminal deletion underwent a brain MRI. Each MRI study consisted of at least axial T1- and T2-, and sagittal T1-weighted images and was evaluated by a neuroradiologist. In two patients a delay in myelination was seen, including patient 29 with the smallest deletion (69.9-76.0 Mb), a region which includes the recently suggested critical region for myelination in 18q23. [26,27]

***Mental retardation***

Mental retardation can be classified based on severity, represented by a Full Scale Intelligence Quotient: mild (IQ=50-70); moderate (IQ=35-50); severe (IQ=20-35) and profound (IQ=0-20). [31] All patients of our study cohort were tested for mental retardation at various ages. Most commonly used tests were the Bayley Scales of Infant Behavior-II, the Wechsler Intelligence Scale for Children-Revised (WISC-R) and the Wechsler Adult Intelligence Scale III.

Most 18q deletion patients in our cohort were mentally retarded, although the level of mental retardation (MR) ranged from very mild to severe. Patients 1-7 all had severe MR, patients 8-19 had various levels of MR and patient 20 to 29 all showed mild or no mental retardation. Therefore, a critical region for mental development could be located proximal from 18q21.33 (25.2-61.4 Mb). Deletions located distal to 18q21.33 did not consistently cause MR and when present, the retardation was usually very mild.



***Other, less known features***

Seventeen patients were assessed for immunoglobulin A (IgA) deficiency. In two patients, number 7 and 17, a deficiency was detected, suggesting that a gene(s) for IgA production could be located distal to band 18q21.32 (58.1-76.0 Mb). However, a high number of patients with overlapping deletions displayed normal IgA levels.

Other common features in our group of patients were obesity, hyperlaxity, strabismus, eczema and behavioral problems. However, no clear genotype-phenotype correlations could be established for these traits. An overview for all phenotypic traits for which a genotypic correlation was established is shown in Figure 4.1.2.

## DISCUSSION

Patients with deletions of the long arm of chromosome 18 display a wide variety of phenotypic traits. In order to correlate these phenotypic traits with the chromosome 18q abnormalities, we assessed molecular karyotyping results obtained by array CGH and clinical features in a cohort of 29 patients with an 18q deletion. This is the first study in which the tiling path array CGH technique has been used to exactly determine the size and position of the 18q deletion in such a large number of patients.

In all 29 patient samples different breakpoints were detected, thereby confirming previously published data that there is no breakage hotspot involved in 18q deletions. [7]

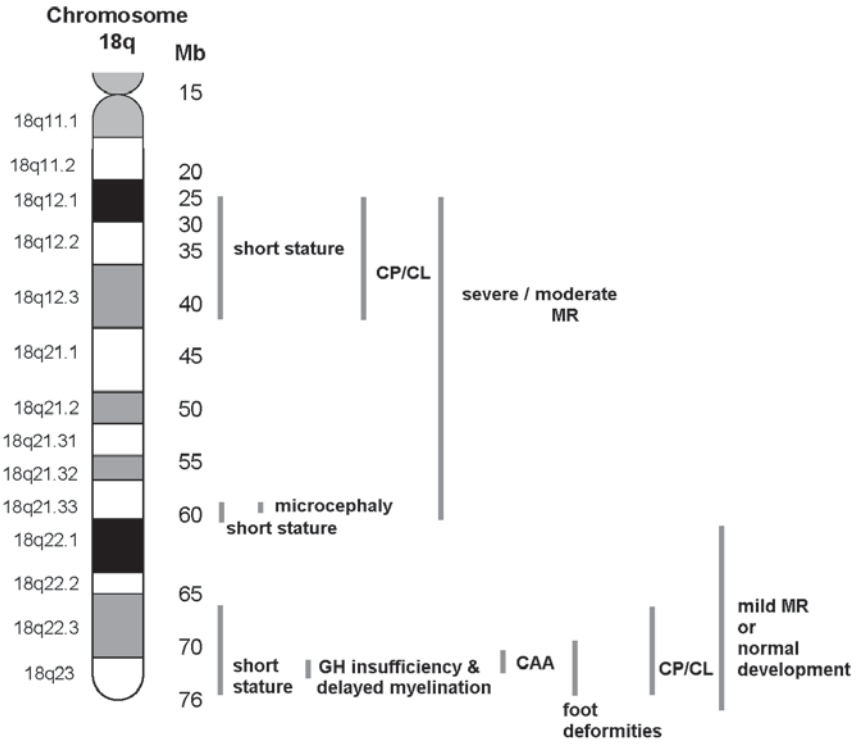


**Figure 4.1.2** A new phenotypic map of chromosome 18q indicating the critical regions for various clinical features. CAA, congenital aural atresia; CP/CL, cleft palate/cleft lip; MR, mental retardation.

In 1993, Kline *et al.* studied seven patients with an 18q deletion. [6] These authors defined a critical region for microcephaly to be located between 18q21.2 and 18q21.3. Our data are in conformity with these results and further refine the critical region to a 1 Mb segment in band 18q21.33 (58.5-59.5 Mb).

Within this region, seven known genes are located including *BCL2*, *FVT1*, *VPS4B* and four members of the serpin B family (UCSC Genome Browser, release March 2006, http://genome.ucsc.edu/cgi-bin/hgGateway). Whether any of these genes may cause microcephaly is currently unknown. The deleted region in patient 6 does not overlap with the 1 Mb region, however, the microcephaly in this patient can be explained by the complexity of his chromosome aberration.

Short stature was seen in 19 out of 29 patients (66%) in our cohort. This is in accordance with the frequency of 64% reported in a systematic review on growth abnormalities in 18q deletion

patients. [22] Our data confirm and further delineate the previously suggested terminal 18q22.3-qter region by Strathdee and colleagues [7] to 67.7-74.9 Mb. Furthermore, we identified two interstitially located regions for short stature: 18q12.1-q12.3 (25.2-42.9 Mb) and 18q21.32-q21.33 (58.5-61.3 Mb). Recently, a study on 14 patients with an 18q deletion was published [26] including a patient with a deletion 18q21q22.3 (46.7-69.8 Mb) and a stature of -4 SD below the mean. Two of our suggested critical regions for short stature are located within this deletion, thereby supporting the notion that these regions are involved in the development of body height.

A suggested factor involved in growth failure in patients with a terminal 18q deletion is growth hormone deficiency. [32-34] Several years ago, the group of Cody proposed a ~2 Mb critical region for growth hormone insufficiency located on 18q23 (72-74 Mb). [35] Among the genes located in this region are Myelin Basic Protein (*MBP*) and a galanin receptor (*GALR1*). The latter is involved in growth hormone response and is therefore a good candidate gene for growth hormone insufficiency. In our group only two patients underwent growth hormone provocative testing and one of them (patient 18) showed growth hormone insufficiency. Her deletion includes the proposed 2 Mb critical region.



Interestingly, the growth hormone deficiency critical region appears to exactly overlap with a region previously suggested for delayed cerebral myelination. [27,28] As *MBP* plays a considerable role in the formation and maintenance of CNS myelin [36], this gene has been put forward as the primary candidate gene in delayed myelination in 18q- patients. [27,30,37]

Recently, a study on the beneficial effects of growth hormone therapy on the cognitive function of children with terminal 18q deletions was published [38], recommending that all children with an 18q deletion should be carefully examined for short stature and growth hormone levels in order to search for the causative mechanism and to provide optimal care. In contrast to short stature in terminal deletions, no possible explanation for short stature in patients with proximal interstitial 18q deletions has been postulated yet, although disorders within the growth hormone pathway could play a role here as well.

In our previous study on a part of this cohort, we found that the critical region for congenital aural atresia (CAA) was located on 18q22.3-q23 (70-76 Mb). [15] Our current results on 11 additional patients are consistent with these findings and in addition the terminal side of the region was restricted to 74.9 Mb. Recently, this CAA critical region was delineated to a 2.3 Mb region on 18q22.3 (70.6-73.2 Mb) by Dostal and colleagues. [39] This region contains nine known genes and the authors postulated *ZNF407* to be the most interesting candidate gene because of its high conservation and expression in bone tissue.

A less frequently described feature in 18q- patients is cleft palate with or without cleft lip (CP/CL). [24,40] In our cohort, patient 2 defines the proximal critical region for CP/CL: 18q12.1-q12.3 (25.2-42.9 Mb) and patient 6 the distally located critical region: 18q22.3-q23 (67.7-74.9 Mb). An isolated cleft lip was not seen and, interestingly, has never been described in patients with an 18q deletion.

The level of mental development varies among patients with 18q deletions, ranging from severe mental retardation to normal development. [1,7,41] In our cohort of patients, the level of mental retardation was severe in all patients with an interstitial deletion, thus including patient 6 with a terminally located interstitial deletion. However, the severe MR in this patient can be explained by the complexity of the aberration, involving a large duplication of 18p and a deletion of 7q as well. Patients 20-29, who carried a terminal deletion, were all mildly mentally retarded or had a normal cognitive development. We therefore propose a critical region for mental development to be located proximal from 18q21.33 (25.2-61.4 Mb). The region 18q21.33-qter (61.4-76.0 Mb) is associated with mild MR or even normal mental development.

Taken together, the combination of our data and the data from the literature allows for an update of the genotype-phenotype map for 18q (Figure 4.1.2). This map shows that proximal and terminal deletions of chromosome 18q relate to clearly distinct clinical phenotypes.

The key features of De Grouchy syndrome include short stature, delayed myelination, congenital aural atresia, foot deformities and a characteristic facial appearance including midface hypoplasia, hypertelorism, low set malformed ears and a carp shaped mouth. Based on data of previous studies and the present study, all these features can be mapped to chromosome bands 18q22.3 and 18q23, locating the critical region of the typical De Grouchy syndrome to 70.6-74.9 Mb. However, there are different critical sub-regions defined for the various clinical features and the entire region contains over 8 known and 3 putative genes. Further studies are needed to determine whether the terminal 18q deletion syndrome is a single gene disorder or should be considered as a true contiguous gene deletion syndrome.

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chapter 4.2

# Neuropsychiatry and deletions of 18q; case report and diagnostic considerations

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## ABSTRACT

The 18q deletion syndrome can be caused by several terminal and interstitial deletions of which terminal deletions of the distal part of 18q are the most frequent and known as the De Grouchy syndrome. The neuropsychiatric phenotype is not well documented and includes disorganised and disinhibited behaviours as well as language difficulties. Non development of language seems to be specific for cases with a more proximally located interstitial deletion. In the present paper a 18-year-old severely mentally retarded male with an interstitial deletion of 18q is described (46.XY,del(18)(q12.1q21.1)dn) who was referred for behavioural problems and neuropsychiatric evaluation. No categorical psychiatric diagnosis could be established. Given this and other reports, it is advocated to describe the psychopathological phenotype of 18q deletions in a dimensional way that will result in a clinical picture characterised mainly by symptoms from the motor and motivation domains. Treatment should include primarily behavioural measures, combined if necessary with symptomatic psycho-pharmacotherapy.

## INTRODUCTION

Since the first description of a deletion of the long arm of chromosome 18 by De Grouchy and co-workers in 1964, patients with deletions of 18q have been reported relatively frequent. [1] Its prevalence is estimated to be 1/40,000. Several types of deletions have been described and it has been demonstrated that each individual may have a unique deletion. [2] The majority of the patients has a deletion of the distal part of 18q including region q22q23 and they present with a great variety of somatic anomalies of which hearing loss [3,4], endocrine abnormalities [5,6], immunological dysfunctions [7-9], epilepsy [10] and dysmyelinisation [11] are the most frequent findings. In addition, facial dysmorphisms, hypotonia, short stature and a variable degree of mental retardation are found. [12] The neuropsychiatric phenotype is not well documented and includes impulsivity, temper tantrums, lack of social reciprocity sometimes called autism, obsessive compulsive behaviour, language difficulties and incidentally psychotic symptoms. For post pubescent patients impulsivity, aggressiveness and temper outbursts are the most commonly reported behaviour problems (review reference 12). Interstitial deletions are less frequent and affect either more proximal parts [13,14] or imply a fragment of the distal region of the long arm of the chromosome. These conditions are generally accompanied by a moderate to severe mental retardation and the distally located interstitial deletions may be expected to manifest a subset of the characteristics of the 18q- syndrome. [15,16]



In the present study, we describe a young adult male with an interstitial deletion of chromosome 18 (q12.1q21.1) who was referred because of behavioural problems for which neuropsychiatric evaluation was requested.

## CASE REPORT

The patient is an 18 years old severely mentally retarded male who is the second child from non-consanguineous parents. He was born premature at 34 weeks gestation by caesarean section and had a birth weight of 1965 grams. Because of bradycardia artificial ventilation was necessary directly postnatal. In addition, subluxation of the right hip was present and he suffered from feeding problems due to a cleft palate that was surgically corrected at the age of two. During his first year, the patient suffered from recurrent upper airway infections and fever-induced seizures. His developmental milestones were markedly delayed and he did not develop active language skills. From the age of 10, the patient showed progressive hyperactive and chaotic behaviour with choreoathetotic movements, distractibility and stereotypic

behavioural sequences. EEG registration did not show epileptic features. He was treated for behavioural control with carbamazepine for some months without any result. Subsequently, a diagnosis of attention deficit hyperactivity disorder was considered and treatment with methylphenidate was started. Despite chronic treatment with this psychostimulant, no behavioural improvement was achieved. In addition, an autistic disorder was supposed. Both psychiatric diagnoses were proposed elsewhere by a general practitioner. At age 15 chromosome analysis was performed and demonstrated an interstitial deletion of the long arm of chromosome 18: 46,XY,del(18)(q12.1q21.1)dn (Figure 4.2.1). The breakpoints were confirmed by array CGH analysis (Figure 4.2.2).

Physical examination revealed a height of 161 cm (-2.5 SDs), a bodyweight of 54 kg (+1.0 SDs corrected for height) and a head circumference of 58 cm (+0.5 SDs). Biochemical tests, including endocrine parameters (GH, T4, T3 and TSH) showed no abnormalities. His phenotype was characterized by a flat midface, epicanthal folds, dysmorphic and low set ears (Figure 4.2.3), scoliosis (most likely the result of hip subluxation) and cryptorchidism. At examination, the patient presented with hyperactive, poorly goal-oriented, over reactive and chaotic behaviour, badly coordinated movements, severe distractibility and several forms of stereotyped behaviour. He did not show expressive language and his receptive language was poor. The patient actively sought physical contact showing a capacity for emotional interactions without, however, any insight, self-appraisal or signs of differentiated mental processes. There were no abnormalities in the regulation of mood and anxiety and no

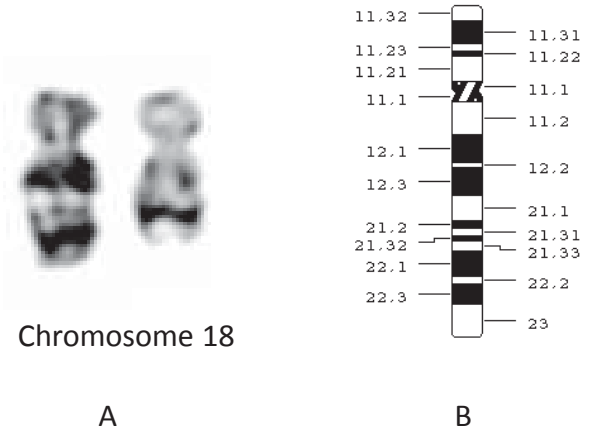


**Figure 4.2.1** **(A)** Both chromosomes 18 of the patient are pictured, with the right chromosome showing the interstitial deletion (18)(q12.1q22.1). **(B)** The ideogram of chromosome 18.

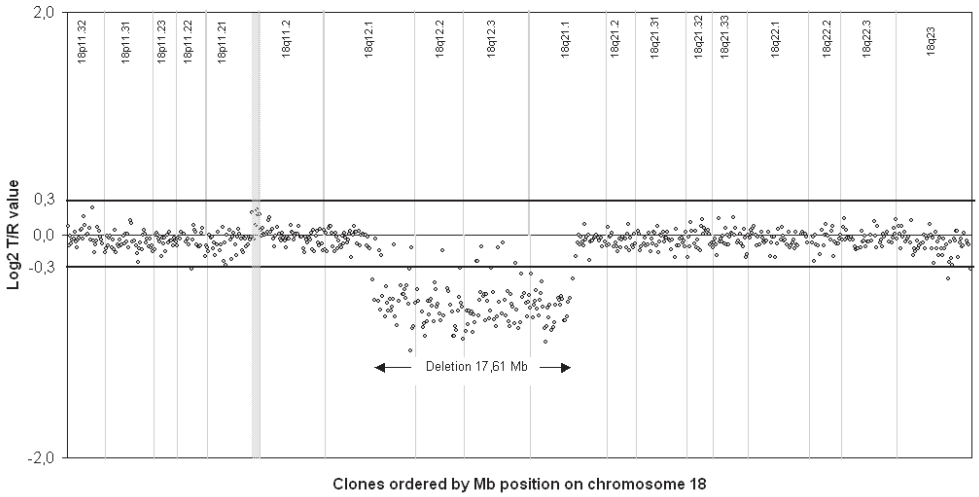


**Figure 4.2.2** The array was composed of 815 cloned chromosome 18 genomic DNA targets, ordered from pter to qter on the basis of mapping positions obtained from the May 2004 freeze of the UCSC genome browser (http://genome.ucsc.edu/). The circles represent the mean log2 test-over-reference (T/R) intensity ratios for this patient, showing a deletion of 17.61 Mb genomic sequence. Note that the vertical lines represent the boundaries of the chromosome 18 banding pattern. The vertical lines at Log2 T/R ratios of 0.3 and -0.3 indicate the thresholds for copy number gain and copy number loss, respectively.



**Figure 4.2.3** The patient at the age of 16 years.

signs of bizarre, incomprehensible behaviour suggestive for psychotic experiences. Clinical manifestations of epilepsy were absent. Screening of cognitive functions and assessment of IQ were not possible.

Although his behaviour over time appeared to be highly determined by external contingencies, a treatment with valproic acid was started. After one year of treatment with this anticonvulsant (dose: 700 mg daily; plasma concentration: 60 to 80 mg/l), no improvement was observed. Subsequently a behavioural treatment was applied and the patient was treated symptomatically with twice-daily 1 mg risperidone (plasmaconcentrations risperidone and hydoxyrisperidone: 10 and 9 µgr/l), which resulted in a substantial diminuation of his hyperactive behaviour.

## DISCUSSION

In this case report the neuropsychiatric profile of a severe mentally retarded young male with a de novo interstitial deletion of the long arm of chromosome 18 is described. His behavioural phenotype is characterized by a non-specific profile of behaviours, the absence of expressive language and poorly developed receptive verbal comprehension as well as by a desire for physical contact in the absence of insight, self-appraisal and differentiated mental processes. So far, only four patients with a comparable interstitial deletion have been reported. [7,15,17] Other publications deal either with more proximal interstitial deletions [13,14,17,18] or with terminal deletions due to different unbalanced translocations. [7,19,20] Most reports, however, describe patients with a terminal deletion. Terminal and distal deletions including the 18q22q23 region result in the De Grouchy phenotype including mild to moderate mental retardation, short stature, hypertelorism, narrow or atretic external ear canals, typical shape of the ear with prominent crus helix, small hands with proximally implanted thumbs, club feet and eczema. The described patient has a deletion 18q12.1q21.1, not including the region responsible for the typical De Grouchy phenotype. Although he has features that are also found in De Grouchy syndrome like short stature and cleft palate, he lacks the characteristic ears and hands and has a more severe mental retardation than usually seen in De Grouchy syndrome.

With respect to the limited number of cases with a proximal interstitial deletion, the clinical pattern comprises mild dysmorphic features, mental retardation, lack of major malformations, epilepsy and 'behavioural abnormalities' such as impulsivity, irritability, hyperactivity and increased distractibility. [17,18]

In patients with terminal deletions a possible relationship has been demonstrated between the extent of the deletion and the number/size of somatic anomalies. [2,21]

No relationship could be established between the deletion size and measures of cognition and behaviour. [12] Although not mentioned in all reports, a more proximally located interstitial deletion may be associated with non-development of expressive language.

According to the literature, the neuropsychiatric profile of 18q- syndrome is characterized by disinhibited and maladaptive behaviours. In some cases, autistic-like features are described that, however, may be related to the level of cognitive functioning. [12,22] Despite the suggested genetic linkage between bipolar affective disorder and the q21-23 region of chromosome 18 [23], no cases with fluctuating affective symptoms have been reported. In the described patient, the behavioural abnormalities were previously attributed to an autistic or an attention deficit hyperactivity disorder. His symptom profile, however, does not meet the diagnostic criteria for either of these categories.



With respect to the assessment of neuropsychiatric symptoms in mentally retarded patients it has to be emphasized that the current practice to establish categorical psychiatric diagnoses is not appropriate and even unsuitable since they do not account for the highly variable developmental history, the level of cognitive and emotional functioning and the great variation in the phenotypical expression of mental and behavioural symptoms. It is therefore advocated to delineate related symptom clusters because they reflect the real world and may point to treatment options and prognosis. [24-26] Although the literature on 18q deletions does not allow constructing a concise psychopathological phenotype, it is of importance that no symptoms from the affective, anxiety and psychotic domains are described in the various case reports. Like in this case, the clinical picture is mainly characterised by symptoms from the motor (impulsivity, distractibility and disinhibition) and motivation (dysexecutive signs) domains. These clinical characteristics exclude a treatment with antidepressants or mood stabilisers and indicate the utility of behavioural measures that can be combined with a symptomatic treatment to reduce disinhibited behaviours.

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chapter 4.3

# Cardiac anomalies in individuals with the 18q deletion syndrome

## Report of a child with Ebstein anomaly and review of the literature

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## ABSTRACT

Individuals with the 18q deletion syndrome are presented with various clinical characteristics, including cardiac anomalies in 24 to 36% of the reported cases. Nonetheless, genotype-phenotype correlations for cardiac anomalies in the 18q deletion syndrome have rarely been reported. We report on two girls with a terminal 18q deletion, one in whom an Ebstein anomaly and Wolf-Parkinson-White syndrome were detected and the other with multiple valve stenosis and a ventricular septal defect. The genotype and cardiac abnormalities of these girls and 17 other individuals with a *de novo* 18qter deletion reported in the literature are reviewed. All 19 individuals shared a small overlapping deletion region between 18q22.3q23. The most common cardiac defects detected were pulmonary valve anomalies and atrial septal defects. Ebstein anomaly, a rare cardiac malformation, was diagnosed in two individuals. Additional molecularly based genotype-phenotype studies are needed in order to pinpoint candidate genes within this region that contribute to normal cardiac development. A careful cardiac evaluation consisting of physical examination, ECG and ultrasound examination should be performed in all individuals diagnosed with the 18q deletion syndrome.

## INTRODUCTION

Within the group of individuals with a rare chromosome abnormality, deletions of the long arm of chromosome 18 occur relatively frequently with an incidence of 1 in 40,000 live births. [1] In 1964, De Grouchy for the first time described an individual with an 18q deletion, stating that she displayed a wide range of features including short stature, characteristic facial features, malformation of the ears suggestive of congenital aural atresia, edema of the foot and severe intellectual disability. [2] Since then, more than 100 individuals have been described. [1,3] The majority of these individuals carry a microscopically visible terminal 18q deletion. Individuals with a distal 18q deletion display phenotypic variation, the most common features being short stature, intellectual disability, characteristic facial dysmorphisms, cleft lip/palate, delayed myelination, foot deformities and congenital aural atresia. [1,3]

The reported incidence of cardiac abnormalities in individuals with 18qter deletions is between 24 and 36%. [1,4] Cody *et al*. reported a cardiac anomaly in 24% out of 42 individuals with breakpoints between 18q21 and 18q23. The cardiac anomalies reported in that study were atrial septal defect, ventricular septal defect, pulmonary stenosis and total anomalous pulmonary venous return. No single cardiac defect was predominant. [1]



In this article we describe two new patients with a *de novo*, terminal 18q deletion and a cardiac defect. One patient (individual 1) has severe stenosis of the pulmonary valve, aortic valve, and tricuspidalis, as well as a ventricular septal defect. The other patient, individual 11, has an Ebstein anomaly, which is a condition where the tricuspid valves are displaced into the right ventricle and because of tricuspid valve displacement, the right ventricle may show arterializations and a significant loss of function. The severity of Ebstein anomaly varies widely and it has only once been reported before to be associated with the 18q deletion syndrome. [5] She was also diagnosed with Wolf- Parkinson-White (WPW) syndrome, which has not previously been associated with an 18q deletion.

Furthermore, we reviewed the literature for 18q deletion patients with cardiac anomalies and we reevaluated previously described individuals known in our centre. In total 19 persons with a distal 18q deletion and a heart defect were included in this review: two girls described in this clinical report and 17 individuals previously reported in the literature, including three individuals from our centre.

## CLINICAL REPORT

The first patient (individual 1 in Table 4.3.1) was born after an unremarkable pregnancy by caesarean section due to fetal distress at $41^{+5}$ weeks of gestation with a birth weight of 2,460 grams (SDS < -2.5). This girl was the first child of healthy, non-consanguineous parents of Caucasian origin. Family history revealed no abnormalities.

Due to respiratory failure the child was intubated and ventilated. Although she was ventilated with 100% of oxygen the transcutaneous arterial oxygen saturation never reached more than 70%.

Clinical examination showed dysmorphic ears and severe hypotonia. A loud heart murmur was heard. Cardiac ultrasound showed severe biventricular hypertrophic cardiomyopathy, severe pulmonary valve stenosis with a gradient of 90 mm Hg, severe aortic valve stenosis with a transaortic gradient of 78 mm Hg, tricuspidalis stenosis and ventricular septal defect. A terminal 18q21.2 deletion was detected by routine karyotyping. As the parental karyotypes were both normal, the 18q21.2qter deletion had occurred *de novo* in this girl. At the age of 11 days she died due to respiratory failure and inoperability of the cardiac anomaly.

The second patient (individual 11 in Table 4.3.1) was born after an unremarkable pregnancy and normal vaginal delivery at 39 weeks of gestation with a birth weight of 3,200 grams (SDS -0.5). She was the fourth child of healthy, non-consanguineous parents of Caucasian origin. The mother was a 40-year-old woman at the time of delivery. Family history revealed no abnormalities.

Clinical examination showed hypotonia, bilateral club feet and dysmorphic ears. No heart murmur was heard. Drinking difficulties occurred probably due to the hypotonia and a poor sucking reflex. A terminal 18q21.33 deletion was detected by routine karyotyping. As the parental karyotypes were both normal, the 18q21.33-qter deletion had occurred *de novo* in this girl.

At the age of 18 months her psychomotor development was delayed. She was functioning at the level of a nine-month-old infant. A brain MRI showed a delay in myelination. Furthermore, bilateral congenital aural atresia and as a consequence a hearing loss of 70 dB was detected, for which she received a Bone-Anchored Hearing Aid (BAHA). At the age of 5.5 years a well speaking girl with a normal height of 112.3 cm (SDS -0.5) and a weight of 19.9 kg (SDS 0) was seen. No cardiac murmur was noticed.

At a regular outpatient clinic control at the age of 7.5 years, she complained of tiredness and tachycardia. Clinical evaluation showed, apart from the previously described features of the 18q deletion syndrome, a tachycard (130 beats per minute) (bpm) girl with a high pitched systolic heart murmur at the left sternal border. There were no signs of cardiac failure. An ECG showed a regular sinus tachycardia (130 bpm) with a short PQ-time, delta waves and widened QRS complexes consistent with Wolf-Parkinson-White syndrome. Cardiac ultrasound showed an Ebstein anomaly with tricuspid valve insufficiency, right atrium enlargement and a high pressure in the right ventricle (42 mm Hg). The 24-hours ECG presented a regular sinus tachycardia, but without any supraventricular tachycardia or atrial flutter, so both cardiac rhythm disorders and decompensation caused by Ebstein anomaly were excluded. Additional laboratory testing showed hyperthyroidism due to Graves' disease. After medical treatment of the hyperthyroidism with methimazole (antithyroid drug) and propanolol (beta-blocker), the tachycardia disappeared, as did the cardiac murmur. As a consequence propanolol medication was stopped.

## MATERIALS AND METHODS



In this study a total of 19 individuals with a *de novo*, terminal 18q deletion and a cardiac anomaly were included to examine the genotype-phenotype correlation, being the two individuals described above and 17 individuals previously reported in the literature, including three individuals from our centre.

In addition to the aforementioned individuals (individuals 1 and 11), three additional individuals (individuals 2, 10 and 12 of the current study) were formerly seen in our department and have previously been published in a series of 29 individuals with the 18q deletion syndrome. [3]

In all five G-banded karyotyping (650 band level) was performed on metaphase spreads from cultured, peripheral blood lymphocytes. Furthermore, additional molecular characterization was performed by tiling resolution chromosome 18 BAC array (individuals 2, 10 and 12) or by high-resolution SNP genotyping with the Affymetrix 250K single nucleotide polymorphism (SNP) array (individuals 1 and 11).

The array analysis was completed on genomic DNA isolated from blood following standard procedures. The SNP array experiments were performed according to the manufacturer's protocols (Affymetrix, Inc., Santa Clara, CA, USA) and data analysis and interpretation was

**Table 4.3.1** Overview of the cytogenetic results and cardiac anomalies in 19 individuals with a terminal 18q deletion

| Individual number and publication (original patient ID in publication) | | Karyotype | Additional molecular method | Deletion breakpoint [a] (Mb position) | Cardiac abnormalities |
|---|---|---|---|---|---|
| 1. | This study | 46,XX,del(18)(q21.2) | 250K SNP-array | 49.3 | Hypertrophic cardiomyopathy, pulmonary valve stenosis, aortic valve stenosis, tricuspidalis stenosis and ventricular septal defect |
| 2. | Feenstra *et al.*, 2007 *(Patient 7)* | 46,XY,del(18)(q21.2) | Tiling resolution chromosome 18 BAC-array | 50.7 | Atrial septal defect |
| 3. | Gunes *et al.*, 2008 | 46,XX,del(18)(q21.2) | FISH (18qter probe) | - | Prolapse of the mitral valve, minimal mitral valve and tricuspid deficiency |
| 4. | Digilio *et al.*, 2011 *(Patient 1)* | 46,XY,del(18)(q21.3) | - | - | Ebstein anomaly, pulmonary stenosis |
| 5. | Versacci *et al.*, 2005 *(Patient 3)* | 46,XX,del(18)(q21.3) | - | - | APV with marked dysplasia and insufficiency of the pulmonary valve leaflets, intact ventricular septum, a large PDA with abnormal origin from the aortic arch, mild to moderate aortic valve stenosis, dilation of the pulmonary arterial trunk and ascending aorta, atrial septal defect |
| 6. | Sturm *et al.*, 2000 | 46,XY,del(18)(q21.3) | - | - | Atrial septal defect, enlargement of the right heart, incomplete right bundle branch block |
| 7. | Silverman *et al.*, 1995 *(PatientTH18)* | 46,del(18)(q21.3) | FISH | 53.5-56.5 | Pulmonary valve insufficiency |
| 8. | Ghidoni *et al.*, 1997 *(Patient 4)* | 46,XX,del(18)(q21.3) | 15 highly polymorphic microsatellite markers | 57.4-61.5 | Pulmonary valve stenosis, ventricular septal defect |
| 9. | Silverman *et al.*, 1995 *(Patient KH18)* | 46,del(18)(q21.3) | FISH | 58.0-61.0 | Ventricular septal defect |

| Individual number and publication (original patient ID in publication) | | Karyotype | Additional molecular method | Deletion breakpoint [a] (Mb position) | Cardiac abnormalities |
|---|---|---|---|---|---|
| 10. | Feenstra *et al.*, 2007 *(Patient 15)* | 46,XY,del(18)(q21.32) | Tiling resolution chromosome 18 BAC-array | 59.2 | Subvalvular aortic stenosis |
| 11. | This study (clinical report) | 46,XX,del(18)(q21.33) | 250K SNP-array | 59.2 | Ebstein anomaly and Wolf-Parkinson-White syndrome |
| 12. | Feenstra *et al.*, 2007 *(patient 19)* | 46,XY,del(18)(21.33) | Tiling resolution chromosome 18 BAC-array | 60.9 | Pulmonary valve stenosis |
| 13. | Spinelli *et al.*, 2011 | 46,XY,del(18)(q22) | - | - | Atrial septal defect |
| 14. | Versacci *et al.*, 2005 *(Patient 2)* | 46,XY,del(18)(q22) | - | - | APV with defective and incompetent pulmonary valve leaflets, moderate to severe dilation of pulmonary arterial trunk and ascending aorta, aortic valve stenosis, intact ventricular septum, large PDA with abnormal origin, and left aortic arch with right descending aorta |
| 15. | Silverman *et al.*, 1999 *(Patient LW18)* | 46,del(18)(q22.1) | FISH | 58.5-61.5 | Atrial septal defect |
| 16. | Ghidoni *et al.*, 1997 *(Patient 5)* | 46,XX,del(18)(q22.2) | 15 highly polymorphic microsatellite markers | 66.8-75.0 | Anomalous pulmonary venous return |
| 17. | Rosenberg *et al.*, 2006 *(patient 2)* | 46,XX,del(18)(q22.3) | Array-CGH, FISH | 69.8 | Total anomalous pulmonary venous return |
| 18. | Ghidoni *et al.*, 1997 *(Patient 2)* | 46,XX,del(18)(q23) | 15 highly polymorphic microsatellite markers | 69.8-72.0 | Mild pulmonary and minimal mitral valve regurgitation, pulmonary valve stenosis |
| 19. | Kohonen-Corish *et al.*, 1996 | 46,XX,del(18)(q23) | FISH, microsatellite marker and RFLP analysis | 72.0-75.1 | Dysplastic pulmonary and tricuspid valves |

[a] Breakpoint (range) based on molecular result (hg19 mapping).

SNP = single nucleotide polymorphism, FISH = fluorescent in situ hybridisation, BAC = Bacterial Artificial Chromosome, CGH = Comparative Genomic Hybridization, RFPL = restriction fragment length polymorphism, APV = absent pulmonary valve, PDA = persistent ductus arteriosus.

done as previously described. [6] The positions of the BAC and SNP array targets were converted to the hg19 (GRCh37) assembly of the human genome.

To the best of our knowledge, 14 other individuals with a pure terminal 18q deletion and a cardiac malformation have been published in the literature. Of these, one individual was investigated by array-CGH and confirmation by FISH was performed (individual 17). Five individuals underwent conventional karyotyping only and in seven cases additional molecular studies including FISH and microsatellite marker analysis were performed. In one case (individual 3) additional FISH was performed, although the 18qter probe used was not informative for the determination of the breakpoint.

To assess an unbiased comparison of the cardiac defects, we only included individuals with a pure deletion of chromosome 18q. Individuals with complex chromosomal rearrangements or additional imbalances were excluded.

## RESULTS

Table 4.3.1 provides an overview of all 19 individuals, including their (molecular) karyotype and cardiac anomaly. A graphic overview of the 18q deletion sizes is given in Figure 4.3.1A.

The girls presented in the clinical reports of this article are listed as individual 1 and 11. Out of the 29 individuals in the study of Feenstra *et al.*, 19 could be classified as a pure distal, non-mosaic 18q deletion and in three out of these 19 individuals (16%) a cardiac anomaly was diagnosed, presented as individual numbers 2, 10 and 12, respectively, in Table 4.3.1.

The breakpoints in 18q in the 19 individuals with a *de novo*, terminal 18q deletion currently described, are all located in the bands between 18q21.2 and 18q23 (Figure 4.3.1A). Only two individuals (18 and 19) were reported with breakpoints located in band 18q23. The remaining 17 individuals carried an extended deletion with the breakpoint located in the various sub bands of 18q21 and 18q22.

Pulmonary valve anomaly is the most common heart defect, occurring in nine out of 19 individuals (47%). Five individuals (26%) are diagnosed with an atrial septal defect, either isolated or as part of a complex cardiac defect. In three individuals (16%) a ventricular septal defect was seen. Furthermore, in three individuals (16%) an aortic valve stenosis was detected. The types of cardiac abnormalities varies widely, as the first individual in this report had a hypertrophic cardiomyopathy, while others had insufficient, absent or stenotic valves, an

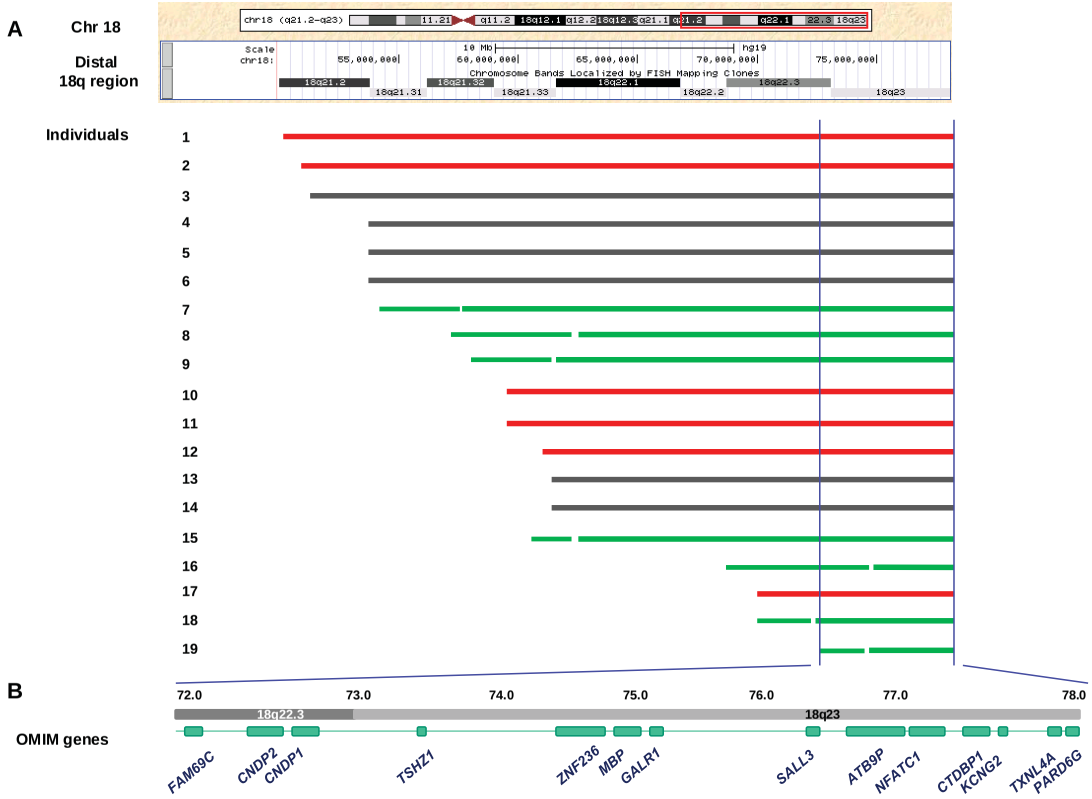


**Figure 4.3.1** Schematic representation of 18q deletions. **(A)** Overview of the 18q deletion sizes of the 19 patients. In the upper part a screen shot of the distal long arm of chromosome 18 in UCSC genome browser, hg19 (http://genome.ucsc.edu/.). The chromosome material deleted in each patient is indicated by a line and the breakpoint ranges by a dotted line. Deletions detected by regular karyotyping are depicted by a grey line, deletions detected by karyotyping and additional molecular cytogenetic analysis by a green line and deletions detected by array analysis are depicted in red. See Table 4.3.1 for additional details of each patient. **(B)** Overview of the OMIM genes located in the overlapping 18q22.3q23 deletion region. Genomic position (in Mb) is shown above and corresponds to human genome reference sequence version hg19.

anomalous pulmonary venous return and the other girl in this clinical report was diagnosed with an Ebstein anomaly and WPW-syndrome. [3-5,7-13]

## DISCUSSION

Cardiac malformations are reported to be diagnosed in 24-36% of the terminal 18q deletion syndrome individuals. [1,4,12] No single cardiac defect is predominantly described, although in the series of Cody *et al*. pulmonary stenosis and atrial septal defects dominated to some extent. [1] In the present series, nine out of the 19 individuals with heart anomalies were diagnosed with pulmonary valve anomalies (47%), which therefore is the most common heart anomaly in individuals with 18q deletion syndrome. An atrial septal defect was detected in five individuals (26%). So our findings are roughly consistent with the results of Cody and colleagues.

To access an unbiased comparison of congenital heart diseases, we only included individuals with a pure terminal deletion of chromosome 18 described in the literature plus the additional five individuals from our centre. Individuals with a complex chromosome 18 rearrangement as well as isopseudodicentric chromosome 18 and mosaic ring chromosome 18 aberrations were excluded from our study and review. The reason for exclusion was that cardiac anomalies detected in these individuals might not only be due to the absence of the distal part of 18q, but this could also be caused or aggravated by the other chromosomal aberration involved. Additional studies are needed to uncover the clinical effects of these complex chromosome aberrations.

All 19 individuals carry a terminal 18q deletion, leading to a critical overlapping region for cardiac anomalies in general in the most distal part of chromosome 18, i.e. 18q22.3q23 (Figure 4.3.1B).

Besides the conventional karyotyping and FISH-analysis, we have used tiling resolution chromosome 18 BAC-array or genome wide 250K SNP-array analysis, both with an average resolution of approximately 200 kb, for a more accurate determination of the breakpoints. All other individuals were mostly investigated before the implementation of the microarray technique and therefore were examined by conventional chromosomal analysis with or without additional FISH and microsatellite marker analysis. Ideally, additional array analysis should be performed in these individuals for an accurate determination of the breakpoints, as correlations between the exact extent of the deletion and cardiac defect could lead to the identification of key genes in cardiac development. The various congenital

heart defects, including rare cardiac abnormalities as anomalous pulmonary venous return, absent pulmonary valve and Ebstein anomaly, suggest a correlation between the terminal 18q deletion syndrome and congenital heart defects. Among the genes located within the commonly deleted 18q22.3q23 region is a highly interesting candidate gene named Nuclear Factor for Activated T-Cells (*NFATC1*; NM_172387). *NFATC1* belongs to the Rel/NF-kb family of transcription factors, which have been implicated in different aspects of embryonic development, transcriptional regulation, cell growth, and apoptosis. In mice, *nfatc1* is expressed in endocardium and is essential for proper valve development. [14,15] It has been shown that a tandem repeat in the intronic region of *NFATC1* is associated with ventricular septal defects. [16] Recently, two heterozygous mutations have been described in a patient with tricuspid atresia. [17]

Another interesting gene within the commonly deleted 18q region is *KCNG2*, a gene encoding a member of the potassium channel, voltage-gated, subfamily G, which may contribute to cardiac action potential repolarization. [18]

In two of the 19 patients described in this article, an Ebstein anomaly was detected, a very rare congenital heart defect. [5] It accounts for less than 1% of all congenital heart diseases and occurs in 1/ 200,000 live births.



About 20 percent of individuals with an Ebstein anomaly have Wolf-Parkinson-White (WPW) syndrome as well. WPW syndrome is characterized by abnormal electrical pathways in the heart, due to an accessory electrical connection between the atrium and ventricle, leading to a disruption of the heart's normal rhythm. Sometimes it leads to delta waves on an electrocardiography, which was also seen in our patient, individual 11. [19]

Since Ebstein anomaly accounts for less than 1% of all congenital heart defects, there might be a significant association between Ebstein anomaly and the 18q deletion syndrome. The finding of Ebstein anomaly in a boy with a terminal 18q21.3 [5] and a girl with a terminal 18q21.33 deletion respectively, suggests the presence of a specific causally related gene located within the distal 18q region. A molecular determination of the 18q21.3 breakpoint in the individual described by Digilio and colleagues could be beneficial in order to compare the exact genotypes of the two individuals with Ebstein anomaly.

Ebstein anomaly has been described in syndromic and non-syndromic individuals. Syndromes associated with Ebstein anomaly are Noonan syndrome, CHARGE syndrome, VACTERL, Kabuki syndrome, Cornelia de Lange syndrome, Ellis-van Creveld syndrome and two

individuals with Holt-Oram syndrome. [5,20,21] Several chromosome aberrations have been described to be associated with Ebstein anomaly, of which 1p36 deletions and 8p23 deletions are most frequently reported. [5,22-26] Others include trisomy 21 [27-29], duplication of the distal long arm of chromosome 15 [30,31], deletion and duplication 11q [32], deletion 10p [33], duplication 9p [34] and a microdeletion in 5q35.1q35.2. [35]

This 5q35.1q35.2 microdeletion encompasses the cardiac transcription factor gene *NKX2-5* and mutations in this gene have been detected in patients with non-syndromic Ebstein anomaly. [36,37] Interestingly, inactivation of *Nkx2-5* in mouse models has shown that this leads to prevention of the nuclear localization of NFATc1 in the endothelial endocardial cells, consequently inactivating its transcriptional functions. [38] The location of *NFATC1* in the critical deleted 18q22.3q23 region together with the interaction of this gene with *NKX2-5* provides evidence that *NFATC1* plays a role in human cardiac development and strongly supports that haploinsufficiency of *NFATC1* can lead to Ebstein anomaly.

Another gene related to non-syndromic Ebstein anomaly is *MYH7*. Heterozygous mutations in *MYH7* cause Ebstein anomaly associated with left ventricular noncompaction cardiomyopathy. [39,40] Patients with non-syndromic Ebstein anomaly have also been screened for mutations in *GATA4*, which maps to chromosome 8p23, but no mutations were found. [5,41]

In conclusion, individuals with a terminal 18q deletion syndrome are at high risk of having a congenital cardiac anomaly. Pulmonary valve anomalies and atrial septal defects are most commonly detected. Therefore, we highly recommend performing a careful cardiac examination consisting of physical examination, ECG and ultrasound examination in all individuals diagnosed with the 18q deletion syndrome. The finding of Ebstein anomaly in two 18q deletion individuals suggests the presence of one or more genes within this chromosome region involved in the etiology of this rare cardiac defect. Our study supports the findings that *NFATC1* plays an important role in human cardiac development and we suggest that disruption of this gene can lead to Ebstein anomaly. Further studies are needed to unravel the exact genotype-phenotype correlation.

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chapter 5.1

# Disruption of the *TCF4* gene in a girl with mental retardation but without the classical Pitt-Hopkins syndrome

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## ABSTRACT

We have characterized a *de novo* balanced translocation t(18;20)(q21.1;q11.2) in a female patient with mild to moderate mental retardation (MR) and minor facial anomalies.

Breakpoint-mapping by fluorescence in situ hybridization indicated that on chromosome 18, the basic helix-loop-helix transcription factor *TCF4* gene is disrupted by the breakpoint. *TCF4* plays a role in cell fate determination and differentiation. Only recently, mutations in this gene have been shown to result in Pitt-Hopkins syndrome (PHS), defined by severe MR, epilepsy, mild growth retardation, microcephaly, daily bouts of hyperventilation starting in infancy, and distinctive facial features with deep-set eyes, broad nasal bridge, and wide mouth with widely spaced teeth.

Breakpoint mapping on the derivative chromosome 20 indicated that here the rearrangement disrupted the chromodomain helicase DNA binding protein 6 (*CHD6*) gene. To date, there is no indication that *CHD6* is involved in disease.

Our study indicates that *TCF4* gene mutations are not always associated with classical PHS but can give rise to a much milder clinical phenotype. Thus, the possibility exists that more patients with a less severe encephalopathy carry a mutation in this gene.

## INTRODUCTION

Mental retardation (MR) is one of the most common disorders, affecting approximately 2% of the general population. The underlying genetic defects are so far largely unknown, and finding the disease genes has important implications for health care and for research into the function of the human brain. During the past few years, we and other groups have shown that one very powerful approach for identifying novel MR genes is the mapping of breakpoints in patients with balanced chromosome rearrangements.

In the ideal situation, the causation between disrupted gene and disease is validated by the presence of additional unrelated patients with a breakpoint in the same gene, as we could recently show for *CDKL5* [1], *SHROOM4* (KIAA1202) [2] and *AUTS2* [3], and/or by the presence of mutations in unrelated patients with MR, for example *CDKL5* [4], *ZNF41* [5], *EHMT1* [6]. Likewise, there are numerous examples documenting that the application of array techniques has enabled the identification of disease-causing DNA copy number changes in patients with MR, including both nonsyndromic [7] and various syndromic forms, for example, CHARGE syndrome [8], the 15q24 [9] and 17q21.31 deletion syndromes [10-12], and 16p13.1 duplications and deletions [13]. Using this technique, mutations in the basic helix-loop-helix transcription factor 4 (*TCF4*) gene, which maps to the long arm of chromosome 18, was recently found to cause a syndromic form of severe MR that was first described in 1978 by Pitt and Hopkins (OMIM 610954). [14-17] In this study, we found *TCF4* truncated by a *de novo* balanced translocation present in a female patient with mild to moderate MR and minor facial anomalies, but not the features of the classical Pitt-Hopkins phenotype.



## MATERIALS AND METHODS

### Cytogenetic analysis and breakpoint mapping by FISH

Samples from the patient and her parents were obtained after informed consent. Chromosome analysis was performed according to standard high resolution methods. Breakpoint analysis was performed by fluorescence in situ hybridization (FISH) experiments on metaphase spreads from the patient lymphoblastoid cell line with YAC, BAC or PAC clones selected from the breakpoint regions. Clones were prepared by standard techniques, labeled with appropriately coupled dUTPs by nick translation or directly labeled by DOP-PCR, and used as probes in FISH as described previously. [18]

### RT-PCR experiments

Total RNAs were isolated from the patient and control lymphoblastoid cell lines using Trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Five micrograms of RNA were used for reverse transcription with Superscript III (Invitrogen), essentially according to the manufacturer's protocol, but in the presence of RNAguard (Amersham Pharmacia, Freiburg, Germany), using random hexamers for priming. Sequences for primers used for PCR and sequencing of TCF4–CDH6 fusion transcripts from der(18) are as follows: CHD6 ex1f 5'-CAA TGG GTC TGT GTT TTG GA-3', TCF4 ex4r 5'-CCC AGG ACC CTG AGC TAC TT-3', TCF4 ex6r 5'-CTG GTG GCA ACC CTG TAA GT-3'.

### Array CGH

Total genomic DNA from the translocation patient was analyzed by array CGH as previously described, [19] using a sub-megabase resolution whole genome tiling path array, consisting of the Human "32k" BAC Re-array set, a series of 32,450 overlapping BAC clones obtained from the BAC/PAC Resources Center at Children's Hospital Oakland Research Institute, the 1 Mb Sanger set, and a set of 390 sub-telomeric clones (assembled by members of COSTB19: Molecular cytogenetics of solid tumors). Copy number gains and losses were determined by a conservative log2 ratio threshold of 0.3 and -0.3, respectively. Profile deviations consisting of three or more neighboring BAC clones were considered genomic aberrations, unless they coincided with a DNA copy number variant as listed in the Database of GenomicVariants (http://projects.tcag.ca/variation/ version December 13, 2005) and/or which was frequently observed in a reference set of more than 800 samples analyzed on the same array platform.

## RESULTS

### Patient description

The patient is the 15-year-old daughter of healthy, unrelated parents. She has an older healthy brother. She was born after an uneventful pregnancy at 41 weeks of gestation and had a birth weight of 3,100 g (-1 SD). During her first year of life she had hypotonia and recurrent infections of the middle ear cavities. At the age of 20 months she was referred to a pediatric neurologist because of developmental delay. Physical examination showed a normal height (85 cm), head circumference (48 cm), and weight (11.5 kg), and minor facial dysmorphisms including a broad,

**Figure 5.1.1** Patient at age 7 years (left) and at 14 years (right). Note the broad, square face, hypertelorism, flat nasal bridge and short neck.

square face, hypertelorism, flat nasal bridge, normally formed but prominent ears and a short neck (Figure 5.1.1). There was a bilateral single palmar crease and her left leg was longer than the right. She had normal neuromuscular tone and normal reflexes.

She could crawl at the age of 16 months and stand at the age of 17 months. She could not speak at the age of 20 months. A cerebral CT scan of the brain showed no abnormalities. A Brainstem Auditory Evoked Potentials (BAEP) examination showed a decrease of sensitivity of both ears at 3 KHz, probably due to cochlear dysfunction.



Follow-up examination at the age of 9 years and 9 months revealed a normal height (140.5 cm), weight (33.6 kg) and head circumference (53.4 cm). The left leg was 4 cm longer than the right. She had mild to moderate MR and attended a school for children with severe learning impairment.

She is now 15 years old. Her height, weight and head circumference are all around the mean for sex and age. The epiphysis of the left knee has been removed because of the significant difference in length of the limbs. She walks easily without stereotypic or abnormal movements. She has had a normal pubertal development. She can speak in sentences, is able to read and write simple text and understands simple tasks. Her behavior is characterized by shyness and lack of initiative. She and her parents reported that she never experienced problems with breathing, such as hyperventilation, and she does not have epilepsy.

## Cytogenetic & molecular analysis

Routine karyotype analysis indicated a *de novo* balanced translocation between the long arms of chromosomes 18 and 20 (t(18;20)(q21.1;q11.2)) (schematically shown in Figure 5.1.2A). Array CGH of total genomic patient DNA did not show any genomic imbalances that might be disease relevant (see Figure 5.1.3). Therefore, we fine-mapped the breakpoints on both derivative chromosomes by FISH using genomic clones from the regions of interest. On chromosome 20, neighboring PAC clones RP4-540H1 (GenBank accession number AL121674) and RP4-661I20 (GenBank accession number AL031669, complete sequence) mapped, respectively proximal and distal to the breakpoint (Figure 5.1.2B). The 5' end of the *CHD6* gene spans most of this region. Therefore, it was likely that *CHD6* was disrupted by the breakpoint (Figure 5.1.2D).

On chromosome 18, BAC clone RP11-409K17 (GenBank accession numbers AC090684, AQ547670, AQ547672) showed hybridization signals on the derivative chromosome 18 but not on derivative chromosome 20 (data not shown), whereas sequence-overlapping clone RP11-619L19 (GenBank accession number AC018994) showed signals on both the derivative 18 and derivative 20 chromosomes (Figure 5.1.2B), demonstrating that it spanned the breakpoint.

FISH with BAC clone RP11-824D7 (GenBank accession number AC090346) revealed that it also spans the breakpoint (data not shown), placing the breakpoint within the overlapping region of approximately 70 kb (Figure 5.1.2C). Interestingly, clone RP11-619L19 lies completely within the *TCF4* gene (also known as SL3-3 enhancer factor SEF2 or E2-2, or immunoglobulin transcription factor ITF2), indicating that this gene is clearly affected by the patient's chromosome rearrangement. Given that both truncated genes are transcribed in the same orientation, it was possible that the chromosome rearrangement resulted in fusion genes. To investigate this possibility further we have combined *TCF4* and *CHD6* specific primers for RT-PCR amplification studies on patient cell line RNA. Sequence analysis of specific products generated with primers located in *CHD6* exon 1 and *TCF4* exon 4 (GenBank accession number NM_001083962), respectively exon 6, indicated that fusion transcripts are produced, with *CHD6* exon 1 spliced to exon 4 (Figure 5.1.4). Therefore, the breakpoint in *TCF4* lies most likely in intron 3 and in *CHD6* it maps within intron 1, upstream of the translational start codon in exon 2.

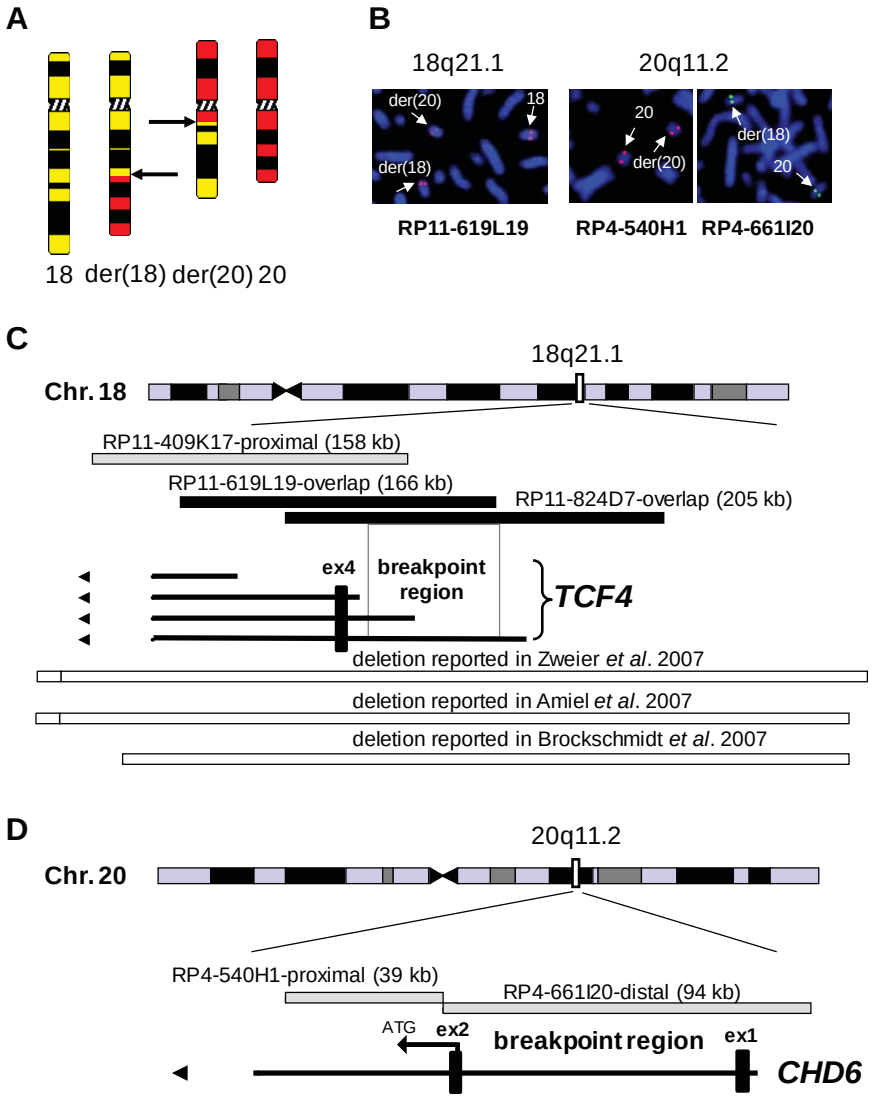


**Figure 5.1.2** **(A)** Ideogram depicting the patient's balanced translocation t(18;20)(q21.1;q11.2). **(B)** Fluorescence in situ hybridization of overlapping clone RP11-619L19 to patient metaphase chromosomes, with signals on chromosome 18 and split signals on both derivative chromosomes 18 and 20 (left panel). Fluorescence in situ hybridization of sequence-overlapping clones with signals on chromosome 20 and derivative chromosome 20 (middle panel), and on chromosome 20 and derivative chromosome 18 (right panel). **(C)** Chromosome 18 breakpoint region showing locations of the proximal clone (grey bar) and overlapping clones (black bars) with respect to the 5' ends of *TCF4* splice variants (arrows) and the deletions reported in patients with the classical Pitt-Hopkins syndrome (open bars). **(D)** Chromosome 20 breakpoint region showing locations of the proximal and distal clones with respect to the *CHD6* gene. In both C and D, the estimated breakpoint locations are depicted.

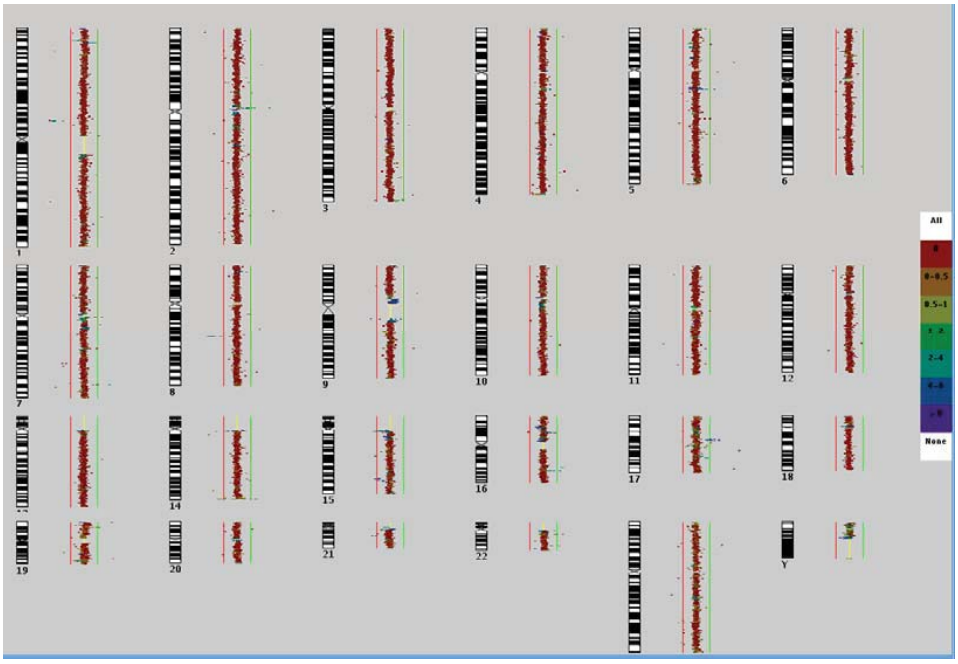

**Figure 5.1.3** Array CGH on patient DNA using a 32k whole human genome tiling path bacterial artificial chromosome (BAC) array as previously described. [19]

*CHD6* untranslated exon 1
GGGAAGGCGGGCCCGAGATGGGTTAGGGTGTCCTTGCAGGGTGTCTGAGCTAAACTT

*TCF4* exons 4-6
CACCAAATAATAGCTGTTTGTATTTTGGCTGCTGCAGGAGCCATTTTAGATGTAGAAGA
CAGAAGTAGCTCAGGGTCCTGGGGGAATGGAGGACATCCAAGCCCGTCCAGGAACTATG
GAGATGGGACTCCCTATGACCACATGACCAGCAGGGACCTTGGGTCACATGACAATCTC
TCTCCACCTTTTGTCAATTCCAGAATACAAAGTAAAACAGAAAGGGGCTCATACTCATC
TTATGGGAGAGAATCAAACTTACAGGGTGGGCCACCAGA

**Figure 5.1.4** Sequence of specific RT-PCR product obtained from patient cell line RNA with primer set CHD6ex1f 5′-CAA TGG GTC TGT GTT TTG GA-3′, TCF4 ex6r 5′-CTG GTG GCA ACC CTG TAA GT-3′. Sequence analysis indicated that in the translocation patient *CHD6* exon 1 (shaded in grey) is perfectly spliced to *TCF4* exon 4.

## DISCUSSION

We have characterized a *de novo* balanced translocation in a patient with MR and mild facial dysmorphisms and identified disrupted genes at each breakpoint. *CHD6* was disrupted by the chromosome 20 breakpoint upstream of the translational start codon. There is so far no indication that this gene plays a role in disease.

Disruption of the *TCF4* gene provides the most likely cause of the clinical phenotype present in the patient. The *TCF4* gene product belongs to the basic helix-loop-helix class of DNA binding proteins and was first found to be a transcriptional activator that binds to glucocorticoid response elements of retrovirus enhancers. [20] Of note, three recent studies have highlighted a role for *TCF4* in the autosomal dominant Pitt-Hopkins syndrome (PHS), which is defined by severe motor retardation and MR, epilepsy, microcephaly, daily bouts of diurnal hyperventilation, short stature, and a distinctive facial appearance. The *TCF4* mutation spectrum includes microdeletions (0.5-1.8 Mb in size) identified by array-comparative genomic hybridization and molecular karyotyping, and missense, stop or splicing mutations. Therefore, the authors suggested that haploinsufficiency of *TCF4* causes PHS. [15-17]

The clinical features present in the translocation patient with disrupted *TCF4* gene only partially overlap with the more complex picture seen in the published PHS patients, including MR and facial anomalies. Overall, the translocation patient we report is much less severely affected. Her MR is mild to moderate, whereas in patients with PHS, the MR is severe, and they cannot speak and have difficulties walking. It is also relevant that so far (at the age of 15 years) the translocation patient showed no epilepsy or breathing abnormalities, which are clinical features frequently present in PHS patients (see Table 5.1.1). In addition, growth parameters are normal in the translocation patient, whereas PHS patients have short stature.

There are several potential explanations for the difference in clinical severity. In the translocation patient the chromosome breakpoint disrupts *TCF4* upstream to exon 4. Therefore, not all splice variants are affected by the rearrangement. In addition, fusion transcripts between *TCF4* and *CHD6* are produced in the patient cell line. These might be translated into truncated *TCF4* protein, which could be partially functional, thereby resulting in a less severe handicap.

These hypotheses are further supported by the observations that PHS patients with missense mutations and microdeletions are similarly severely affected and that in mice, embryonic development is sensitive to basic-helix-loop-helix transcription factor copy number, including *Tcf4*. Recently, it has been shown that Tcf4-/- deficient mice have disrupted pontine nucleus development. [21]

Still another possibility is that mutations in *TCF4* result in a broader clinical spectrum than known to date, ranging from the mild to moderate MR and minor facial anomalies (as seen in our translocation patient) to the severe MR encephalopathy.

**Table 5.1.1** Overview of clinical features in patients with a *TCF4* mutation

| Phenotype | Translocation patient (this study) | Patient 1 Amiel *et al.* [2007] | Patient 2 Amiel *et al.* [2007] | Patient 3 Amiel *et al.* [2007] | Patient 4 Amiel *et al.* [2007] | Patient 1 Zweier *et al.* [2007] | Patient 2 Zweier *et al.* [2007] | Patient 3 Zweier *et al.* [2007] | Patient 4 Zweier *et al.* [2007] | Patient 5 Zweier *et al.* [2007] | Patient 6 Zweier *et al.* [2007] | Patient 1 Brockschmidt *et al.* [2007] | Patients with *TCF4* mutations (*n*=12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | F | F | F | M | M | M | M | M | F | M | F | F | 6M/6F |
| Birth weight | 25-50th | 50th | 50th | 50th | 50th | >3rd | >10th | >25th | 10th | 50th | 3rd | 97th | 3rd-97th |
| HC at birth | NA | 25th | 10th | 50th | 50th | 3rd | 25th | 25th | 10th | NA | NA | 90th | 3rd-90th |
| Current height | 50th | 25th | 25th | 10th | 50th | -3 SD | -3 SD | 10-25th | 25th | <3rd | <3rd | 10th | <3rd-50th |
| Current weight | 50th | 25th | 25th | 25th | 25th | NA | NA | NA | NA | NA | NA | 75-90th | 25th-90th |
| HC at the last examination | 50th | 3rd | <2nd | 3rd | 3rd | -3 SD | -5 SD | 25th | 25th | <3rd | <3rd | 75th | <2nd-75th |
| MR | Mild–moderate | Severe | Severe | Severe | Severe | Severe | Severe | Severe | Severe | Severe | Severe | Severe | 12/12 |
| PHS facial gestalt | + (mild) | + | + | + | + | + | + | + | + | + | + | + | 12/12 |
| Strabismus | - | + | + | + | + | - | - | + | - | + | - | - | 6/12 |
| Single palmar crease | + | + | + | + | + | + | + | + | + | + | NA | + | 11/11 |
| Genitalia | Normal | Normal | Normal | Abnormal | Abnormal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | 2/11 |
| Chronic obstipation | - | + | + | + | + | + | Hirsch-sprung | - | - | - | - | - | 7/12 |
| Brain MRI abnormalities | - (only CT) | + | + | + | NA | + | + | + | + | NA | - (only CT) | - | 7/10 |
| Epilepsy | - | - | + | + | + | + | + | - | - | - | - | - | 5/12 |
| Breathing anomalies | - | - | + | + | + | + | + | - | + | + | + | + | 9/12 |

HC, head circumference, NA, not available, MRI, magnetic resonance imaging, CT, computer tomography.

There are many specific genotype–phenotype correlations in other MR-associated disorders. Several XLMR genes, for example, have now been shown to cause both syndromic and non-syndromic forms of MR (depending on the nature of the mutation), and it is also well established that identical mutations can lead to diverse phenotypes. Our findings suggest that the selection criteria for including patients into future *TCF4* mutation screening should perhaps also include milder clinical phenotypes than the typical clinical picture present in PHS patients. In this context, it is worth noting that the contribution of the second translocation breakpoint, which lies within the *CHD6* gene region, is unclear. It might have an impact on the cochlear dysfunction present in the translocation patient but not observed in other PHS patients. *CHD6* belongs to the family of CHD proteins and might play a role in RNA polymerase II-mediated transcriptional processes.

Taken together, the *TCF4* disruption described here in a girl with MR, in conjunction with published results of *TCF4* mutations in patients with PHS, [15-17] provide clear evidence for *TCF4* playing an important role in the brain. Our observations suggest that not all mutations in this gene cause a severe phenotype that is characteristic for PHS.

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chapter 5.2

# Disruption of Teashirt Zinc Finger Homeobox 1is associated with congenital aural atresia in humans

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## ABSTRACT

Congenital aural atresia (CAA) can occur as an isolated congenital malformation or in the context of a number of monogenic and chromosomal syndromes. CAA is frequently seen in individuals with an 18q deletion, which is characterized by intellectual disability, reduced white-matter myelination, foot deformities, and distinctive facial features. Previous work has indicated that a critical region for CAA is located in 18q22.3.

We studied four individuals (from two families) with CAA and other features suggestive of an 18q deletion, and we detected overlapping microdeletions in 18q22.3 in both families. The minimal region of deletion overlap (72.9-73.4 Mb) contained only one known gene, *TSHZ1*, which was recently shown to be important for murine middle-ear development.

Sequence analysis of the coding exons in *TSHZ1* in a cohort of 11 individuals with isolated, nonsyndromic bilateral CAA revealed two mutations, c.723G>A (p.Trp241X) and c.946_947delinsA (p.Pro316Thr*fs*X16), and both mutations predicted a loss of function.

Together, these results demonstrate that hemizygosity of *TSHZ1* leads to congenital aural atresia as a result of haploinsufficiency.

## INTRODUCTION

Congenital aural atresia (CAA) is a rare malformation of the ear that occurs in approximately 1 in 10,000 live births. [1] It presents unilaterally more often than bilaterally. Its characteristics can vary from a narrow external auditory canal and hypoplasia of the tympanic membrane and middle ear cleft to a complete absence of middle-ear structures and anotia (bony atresia of the external auditory canal and hypoplasia of inner ear structures). In the past, different classifications of CAA have been introduced on the basis of clinical findings. In 1955, Altmann was the first to describe a CAA classification, [1] which has been modified over the years by others. [2-4]

CAA type I is classified by a bony or fibrous atresia of the lateral part of the external auditory canal and an almost normal medial part and middle ear. CAA type II is the most frequent type and is characterized by partial or total aplasia of the external auditory canal. In type IIA, the external auditory canal is either affected by a complete bony atresia of its medial part or partially aplastic, ending blindly in a fistula that leads to a rudimentary tympanic membrane. CAA type IIB is characterized by a bony stenosis of the total length of the external auditory canal. Finally, CAA type III is characterized by bony atresia of the external auditory canal and a very small or absent middle-ear cavity. [2]

CAA might be present as an isolated malformation but is also seen as a feature of complex syndromes such as Crouzon syndrome [MIM 123500], Treacher Collins syndrome [MIM 154500], Townes Brocks syndrome [MIM 107480], and branchiootorenal syndrome [MIM 113650], as well as aneuploidy syndromes including Turner syndrome (45,X) and trisomies 13, 18, and 21. [5-10] Although not fully penetrant, CAA type IIA in the absence of microtia or anotia is most frequently seen in individuals with a deletion of the long arm of chromosome 18 (MIM 601808). [11] In 1964, De Grouchy described individuals with an 18q deletion, stating that they displayed CAA and a wide range of associated features including short stature, characteristic facial features, intellectual disability, and foot deformities. [11,12]



The majority of individuals with an 18q deletion carry a microscopically visible terminal deletion of the long arm of chromosome 18. [13] Yet, in a small subset of individuals, routine cytogenetic studies reveal a normal G banded karyotype.

Several genotype-phenotype studies have been performed in persons with 18q deletions of various sizes so that critical regions corresponding to the different clinical symptoms of the 18q deletion syndrome could be defined. [14-16] These efforts have resulted in overlapping critical regions for white-matter disorders and delayed myelination, growth hormone insufficiency,

foot deformities, and CAA, all nested within the region from 18q22.3 to 18q23. [14-16] Given that multiple genes reside in the region of deletion overlap, it was concluded that further studies would be needed to determine whether the typical 18q deletion syndrome represents a single gene disorder or whether it should be considered a contiguous deletion syndrome. [14] Fine mapping of microscopically visible deletions via molecular techniques, such as a chromosome 18q BAC array, mapped CAA to a 2.3 Mb region in 18q22.3q23 (between markers D18S489 and D18S554). However, it remained unclear whether CAA could be separated from the other common features of the 18q deletion syndrome. [17,18]

Here, we report that isolated CAA and the CAA phenotype in the 18q deletion syndrome are both caused by haploinsufficiency that results from a heterozygous deletion or loss-of-function mutation of *TSHZ1*, whose ortholog is essential for murine middle-ear development. [19]

## PATIENTS, METHODS AND RESULTS

All procedures were performed in accordance with the ethical standards of the Radboud University Nijmegen Medical Centre Ethical Committee. After obtaining informed consent, we evaluated the presence of microdeletions in four individuals (from two different families) who had a phenotype consistent with the 18q deletion syndrome. We used 250K (Nsp1) single-nucleotide polymorphism (SNP) microarrays (Affymetrix, Inc., Santa Clara, CA, USA). [20] SNP microarray experiments were performed according to the manufacturer's instructions. Copynumber estimates were determined with the CNAG software package v2.0, and the genomic locations of the SNP positions were mapped according to the Genome Reference Consortium Human Genome Build 37 (GRCh37).

Individual 1 is the second son from nonconsanguineous parents. He was born at term after an uncomplicated pregnancy and had a normal birth weight. Psychomotor development was delayed: He started walking at 21 months, and at the age of 4 years, a 10-15 month delay in speech and language development was detected. A pure-tone audiogram was performed when the child was cooperative at 4 years of age and showed mild left- and right-sided conductive low-frequency hearing loss of 17 and 22 dB, respectively (Table 5.2.1). At eight years of age, he developed epileptic seizures, and EEG abnormalities occurred after sleep deprivation. He was also noted to have mild intellectual disability (an IQ of 60) and behavioral problems involving autistic features. He was a cooperative, healthy boy with a normal height and head circumference. His dysmorphic features included hypertelorism, an upturned nasal tip, and

**Table 5.2.1** Overview of clinical features in eight individuals with an 18q22.3q23 microdeletion or *TSHZ1* mutation

| Individual | Age (yr) | Intellectual disability | Hearing loss[a] L/R | Type CAA | Other |
|---|---|---|---|---|---|
| 1 (family 1) | 8 | mild | 17 dB/22 dB | narrow external auditory canals | epilepsy, autism |
| 2 (family 2) | 30 | borderline | 60 dB/60 dB | IIA, bilateral | bilateral vertical talus, strabism |
| 3 (family 2) | 5 | normal | 65 dB/65 dB | IIA, bilateral | bilateral vertical talus |
| 4 (family 2) | 1 | mild motor delay | 70 dB/45 dB | IIA, bilateral | bilateral vertical talus |
| 5 (family 3) | 10 | normal | 42 dB/42 dB | IIA, bilateral | no |
| 6 (family 4) | 41 | normal | 40 dB/40 dB | IIA, bilateral | no |
| 7 (family 4) | 12 | normal | 50 dB/55 dB | IIA, bilateral | no |
| 8 (family 4) | 12 | normal | 48 dB/45 dB | IIA, bilateral | no |

Mb, megabase; L, left; R, right; CAA, congenital aural atresia; Type IIA, complete bony atresia of the medial part of the external auditory canal, or the canal is partially aplastic and ends blindly in a fistula that leads to a rudimentary tympanic membrane.

[a] Measurement of air conduction before surgical intervention or use of bone-anchored hearing aid.

a thin upper lip (Figure 5.2.1A). CT scans showed bilateral, narrow external auditory canals. This finding was confirmed by otoscopy, from which only a small part of the tympanic membrane could be visualized (Figure 5.2.3A).

SNP-array analysis of DNA isolated from blood revealed a 4.3 Mb interstitial deletion in 18q22.3q23, extending between the genomic coordinates 69.2 and 73.4 Mb (arr snp 18q22.3q23 [SNP_A-2065000 > SNP_A-184607]x1; Figure 5.2.2A). SNP-array analysis of the phenotypically normal mother revealed no abnormalities. The father was not available for testing.

Individual 2 is a 30-year-old woman, born at 39 weeks gestation after an uneventful pregnancy to unrelated, healthy parents. Directly after birth, bilateral forefoot deformities were noted and classified as congenital vertical talus (Figure 5.2.1B), for which she received surgical treatment.

She could walk from the age of 2 years. At the age of 15 months, she presented with a bilateral conductive 60 dB hearing loss, which was caused by bilateral atresia of the external auditory canals, consistent with CAA type IIA (Table 5.2.1). She received hearing aids and consequently developed speech, although a delay remained. At ten years of age, the aural atresia of her right

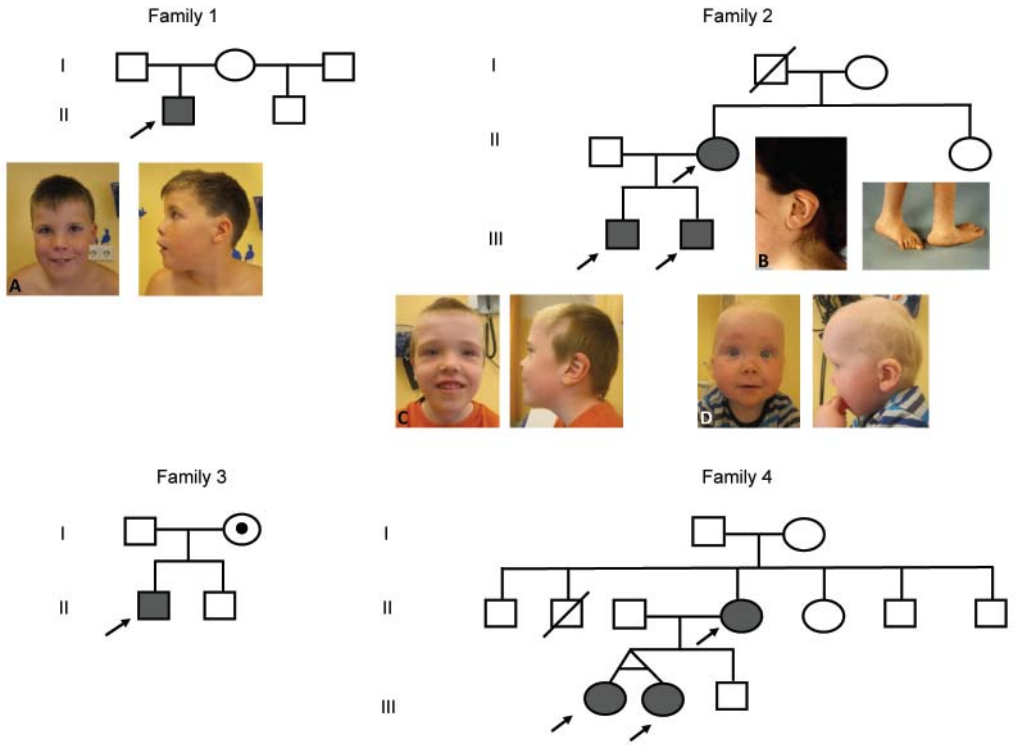


**Figure 5.2.1** Pedigrees and clinical pictures of affected individuals. The individuals with an 18q22.3q23 microdeletion are depicted above (families 1 and 2), and the pedigrees of individuals with a *TSHZ1* mutation are depicted below (families 3 and 4). Note the mild dysmorphic features in individual 1 **(A)**, including hypertelorism, an upturned nasal tip, and a thin upper lip. The mother **(B)** and sons **(C and D)** of family 2 display several features in common, consisting of hypertelorism, down-slanting palpebral fissures, a broad mouth, characteristic low-set ears, and bilateral foot deformities.

ear was successfully operated on, and at the age of 26, she received a boneanchored hearing aid (BAHA) on the left side. Physical examination at the age of 30 years showed a healthy woman with normal height, weight, and head circumference.

Dysmorphic features included hypertelorism, midfacial hypoplasia, and a broad mouth with prominent lips. There was a normal implantation of the ears, which showed a prominent superior crus of the antihelix and underdevelopment of the descending part of the helix (Figure 5.2.1B).

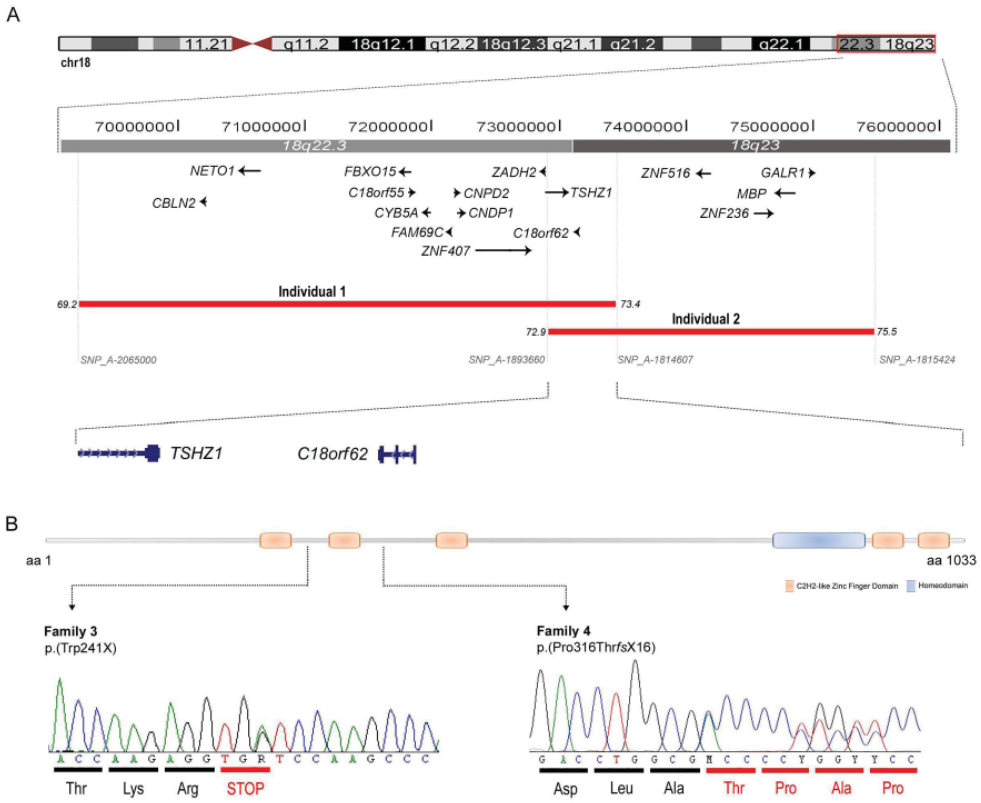


**Figure 5.2.2** Detailed genomic view of 18q22.3q23; organization of *TSHZ1* and mutations detected. **(A)** A schematic representation shows the transcripts mapped to the 18q22.3q23 region. Deletions detected by SNP-array analysis in individual 1 and family 2 are shown by red solid lines, and details on the first and last deleted SNPs are annotated in gray. The shortest region of deletion overlap contains a known gene, *TSHZ1*, and an open reading frame, c18orf62, of unknown function. **(B)** A schematic representation of the *TSHZ1* protein shows its domain structure. Targeted Sanger sequencing of *TSHZ1* in families 3 and 4 revealed mutations in both families; the mutations are shown as partial electropherograms for both families, respectively. Of note, the protein sequence denoted below the electropherograms only shows the mutated sequence.

SNP-array analysis revealed a 2.5 Mb interstitial deletion between the genomic coordinates 72.9 and 75.4 Mb (arr snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]x1; Figure 5.2.2A).

Individual 3 is the first son of individual 2 and her healthy husband. He was born after an uneventful pregnancy at term and had normal birth parameters. Like his mother, he had congenital bilateral vertical talus and bilateral CAA type IIA (Table 5.2.1). Computed tomography (CT) scans showed significant narrowing of the external auditory canals, opacification of the mastoid and middle ear probably related to otitis media, and normal anatomical aspects of the inner ear.

During the first years of life, his hearing was assisted by a bone-conductive hearing aid on a softband. The latter was replaced by a percutaneous titanium screw at the age of 4 3/4 years. Examination at 5 1/2 years of age showed a healthy, cooperative boy with normal height, weight, and head circumference. Dysmorphisms included hypertelorism, mild down-slanting palpebral fissures, a broad mouth, and characteristic low-set ears with a prominent superior crus of the antihelix and hypoplasia of the descending helix (Figure 5.2.1C). SNP-array analysis revealed the same 2.5 Mb interstitial deletion (arr snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]x1) as observed in his affected mother (data not shown).

Individual 4 is the second child of individual 2. A prenatal ultrasound at 20 weeks gestation showed congenital bilateral vertical talus. He was born at term after an otherwise uncomplicated pregnancy and had a normal birth weight. Similar to his mother and older brother, he had congenital bilateral vertical talus and distinctive dysmorphic features including hypertelorism and low-set ears with hypoplasia of the descending helix (Figure 5.2.1D).

Otoscopic examination showed bilateral narrowing of the auditory canals, consistent with CAA type IIA.

During the last examination at the age of 1 year, his parents reported a motor delay. SNP-array analysis revealed the same 2.5 Mb interstitial deletion (arr snp 18q22.3q23 [SNP_A-1893660 > SNP_A-1815424]x1) as observed in his affected mother and brother (data not shown), indicating full cosegregation of the deletion with the phenotype in this family.

Interestingly, the microdeletions in individuals 1 and 2 (and both her sons) showed a 459 kb deletion overlap, which contains one hypothetical protein (C180rf62) and a single known gene, Teashirt Zinc Finger Homeobox 1 (*TSHZ1*; NM_005786.4) (Figure 5.2.2). *TSHZ1* was considered to be a good candidate gene for the observed CAA phenotype on the basis of the deletion overlap and the fact that all four individuals presented with the common

feature of narrow or atretic external auditory canals. This hypothesis was further supported by previously reported Tshz1 loss-of function mutations in mice; these mutations lead to specific malformations of the middle ear components [19] and emphasized the importance of *TSHZ1* in the developing middle ear.

Therefore, conventional bidirectional Sanger sequencing was performed for this specific gene in 11 persons (6 sporadic and 5 familial individuals) with an isolated, bilateral form of CAA type IIA and normally shaped pinnae. In total, four individuals and one unaffected relative showed heterozygous loss-of-function mutations, including a sporadic affected person (individual 5), his unaffected mother, and a family with three affected individuals consisting of a mother and her two daughters (individuals 6-8) (Table 5.2.1).

In individual 5 (family 3), we identified a heterozygous c.723G>A mutation, which was predicted to introduce the premature stop codon p.(Trp241X) (Figure 5.2.2B). This boy was the first child of healthy, nonconsanguineous parents (Figure 5.2.1). He was born at term after an uneventful pregnancy, had normal birth parameters, and no congenital anomalies were detected. He had normal motor development, but impaired speech and delayed language development were noticed between the ages of 3 and 4 years.

Pure-tone audiometry at the age of five demonstrated a 42 dB bilateral conductive hearing loss due to CAA type IIA (Figure 5.2.3B). A BAHA Softband and subsequent percutaneous titanium BAHAs were applied successfully.

Physical examination at the age of 10 years showed no facial dysmorphisms or other features associated with the previously determined critical 18q deletion regions that include *TSHZ1*. Segregation studies revealed that the detected stop mutation was also present in his phenotypically normal mother. Examination of her ears demonstrated no abnormalities to the external auditory canal or to the tympanic membrane or her hearing.



In family 4 (Figure 5.2.1), we identified a frameshift mutation due to a single base pair insertion (c.946_947delinsA), which is predicted to cause the premature stop codon p.Pro316Thr*fs*X16 (Figure 5.2.2B). As expected, this mutation showed an autosomal-dominant segregation pattern. The affected mother of this family (individual 6) had isolated bilateral conductive hearing loss due to CAA type IIA, for which she had bilateral surgical treatment at the age of 3.

Her hearing declined slowly over the following decades, and she recently received a BAHA on the left side at 42 years of age.

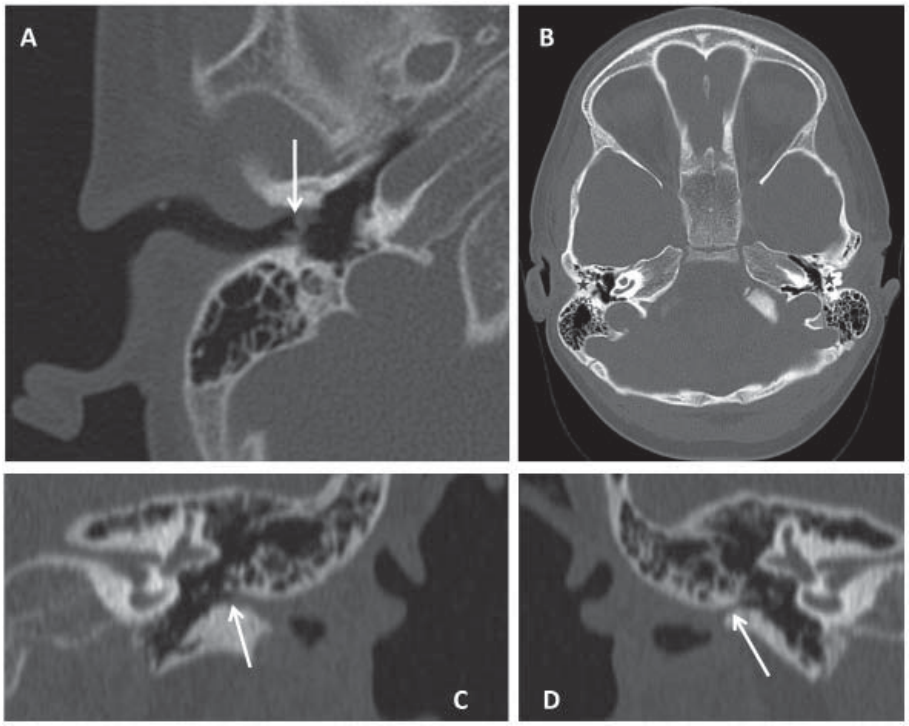


**Figure 5.2.3** CT scan images of individuals with an 18q deletion or *TSHZ1* mutation. An axial CT scan of individual 1 **(A)** shows a narrow external auditory canal with a normal tympanic membrane and a grommet (white arrow) in place of the right ear. **(B)** shows an axial-plane bilateral CT scan of individual 5 with bilateral CAA type IIA (stars). **(C)** shows a coronal CT reconstruction of individual 7, demonstrating a CAA type IIA (arrow) and **(D)** shows a coronal CT reconstruction of individual 8, demonstrating a CAA type IIA (arrow).

Individuals 7 and 8, a monozygotic twin pair, are the daughters of individual 6 and also both displayed CAA type IIA (Figures 5.2.3C and 5.2.3D). They had undergone a canalplasty on one ear, and on the contralateral ear, they wore a bone-anchored hearing aid. The mother and daughters showed no notable dysmorphic facial features or any other abnormalities associated with 18q deletion syndrome. It is worth noting that a sequence analysis of the phenotypically normal maternal parents did not reveal the presence of this frameshift mutation, indicating that the mutation occurred de novo in the index person of this family.

The remaining seven individuals did not show any base pair mutations in the coding sequence of *TSHZ1*. For these individuals, whole-gene deletions were excluded with the Cytogenetics Whole-Genome 2.7M Array (Affymetrix) according to the manufacturer's instructions.

To test the (clinical) specificity of *TSHZ1* mutations, we subsequently tested a cohort of 24 individuals with a unilateral form of CAA type I, IIB, or III (Table 5.2.2). In 10 of the 24 individuals, the CAA phenotype was accompanied by mild to severe developmental malformation of the external ear(s), such as microtia or anotia. Sanger sequencing did not reveal any causal mutations in this cohort.

## DISCUSSION

In previous studies, we proposed, in accordance with reports of other groups, [17] that the critical region for isolated CAA was located on chromosome 18q22.3. [14,18] This region was reported to contain nine candidate genes, yet none of the reports mentioned *TSHZ1* as a potential candidate for CAA.

We detected two small overlapping 18q microdeletions in individuals with CAA as a common feature, narrowing the critical interval for CAA to 72.9-73.4 Mb and establishing *TSHZ1* as a strong candidate for the CAA gene. The subsequent detection of both a nonsense and a frameshift mutation in *TSHZ1* in two families with nonsyndromic CAA clearly shows that hemizygosity of *TSHZ1* indeed leads to isolated CAA through haploinsufficiency. This observation further suggests that other genes in the previously established critical region in 18q22.3 should be more relevant to the intellectual disability, facial dysmorphisms, and foot deformities that are commonly seen in the 18q deletion syndrome.

The members of family 2 (individuals 2, 3, and 4) displayed a collection of features including characteristic facial features, bilateral CAA, and vertical talus. The mother and sons described here very much resembled the phenotype of three males and three females in a family described by Rasmussen in 1979 (MIM 133705). [21] Possibly, hemizygosity of one of the other four genes deleted in individuals 2-4, namely *ZNF516*, *ZNF236* (MIM 604760), *MBP* (MIM 159430), and *GALR1* (MIM 600377), could lead to haploinsufficiency and cause congenital foot deformities like vertical talus. Molecular analysis of the family members described by Rasmussen could provide more insight into the hypothesis that Rasmussen syndrome is caused by a microdeletion, which is identical or at least overlapping with the deletion detected in individuals 2-4 in our study.

*TSHZ1* consists of two exons, of which only exon 2 is coding and has a genomic size of 79 kb. *TSHZ1* is a member of the teashirt-type zinc-finger protein family and encodes putative zinc finger transcription factors that are broadly expressed during mouse embryogenesis. [22]

**Table 5.2.2** Overview of clinical features in the cohort of individuals in which *TSHZ1* sequence analysis revealed no mutations

| Sex | Familiar | CAA type L | CAA type R | Pinnae | Intellectual disability | Other abnormalities |
|---|---|---|---|---|---|---|
| F | - | normal | IIA | normal | - | - |
| F | - | IIA | IIA | normal | - | - |
| M | - | - | IIA | mild dysmorphic ear R | - | - |
| M | - | - | IIA | microtia R | - | - |
| M | - | - | IIA | dysmorphic helix R | - | - |
| F | - | | IIB/III | S-shaped ear R | - | - |
| F | - | IIB/III | IIB/III | S-shaped ears | - | - |
| M | - | IIA | III | normal ear L, microtia R | - | - |
| M | - | IIA | - | normal | - | dextrocardia, mildly asymmetric face |
| F | + | - | IIA | normal | - | - |
| M | - | - | IIA | normal | - | - |
| M | + | - | IIB/III | complete anotia R | - | - |
| M | - | IIB/III | IIB/III | bilateral microtia | - | - |
| M | - | - | IIA | normal | - | - |
| F | - | III | - | microtia L | - | - |
| M | - | IIA | - | mild dysmorphic ear L | - | - |
| F | - | - | IIA | normal | - | - |
| M | - | - | IIA | normal | - | - |
| F | + | IIA | IIA | normal | - | - |
| M | + | IIA | IIA | normal | - | - |
| M | - | - | IIA | mild microtia R | - | - |
| F | - | narrow | - | mild dysmorphic ear L | - | - |
| F | - | - | IIA | normal | - | - |
| F | + | I | - | small ear L | - | - |

F = female, M = male, - = absent, + = present, CAA = congenital aural atresia, Type I = bony or fibrous atresia of the lateral part of the external auditory canal and an almost normal medial part and middle ear, Type IIA = complete bony atresia of the medial part of the external auditory canal, or the canal is partially aplastic and ends blindly in a fistula that leads to a rudimentary tympanic membrane,Type IIB = bony stenosis of the total length of the external auditory canal, Type III = bony atresia of the external auditory canal and a very small or absent middle ear cavity.

In vertebrates, three *TSHZ1*-related genes (*TSHZ1*, *TSHZ2* [MIM 614118], and *TSHZ3* [MIM 614119]) have been isolated on the basis of sequence homology.

Recently, knockout mice have been generated for *Tshz1*. [19] *Tshz1* inactivation in mice leads to neonatal lethality and causes multiple developmental abnormalities, including a severe middle-ear phenotype that mimics defects observed in individuals with isolated CAA. In addition, *Tshz1*-deficient mice show a defect of the soft palate, a feature which was not seen in any of the individuals in the current study. The phenotype of *Tshz1*-deficient mice resembles the phenotype seen in *Hoxa3* and *Sall3* mouse mutants. [23,24] Interestingly, the gene families to which these genes belong – the Hox and Spalt gene families – genetically interact with teashirt (*tsh*) in *Drosophila*. [25] Possibly, mutations in genes of these families give rise to a similar CAA phenotype, either isolated or as part of a more complex syndrome. Possible candidate genes might include other members of the teashirt zinc-finger protein family, such as *TSHZ2* and *TSHZ3*, and members of the human HOX and SPALT families. A member of the HOX family, *HOXA2* (MIM 604685), has indeed been described as playing a crucial role in auditory-system malformations, more specifically in an autosomal-recessive form of bilateral microtia, hearing impairment, and partial cleft palate. [26] Similar to what we now observe for *TSHZ1*, the human phenotype caused by *HOXA2* mutations is in concordance with that of the *Hoxa2* knockout mouse. [27]

However, DNA-sequence analysis of *HOXA2* in individuals with isolated microtia did not reveal mutations. [28] In the same fashion, not all individuals with an isolated form of CAA type IIA selected for the present study showed mutations in *TSHZ1*; this finding is in line with the fact that CAA is observed as an endophenotype in multiple syndromes, thereby suggesting that several CAA genes still await discovery.



Most individuals with a terminal 18q deletion have been diagnosed with CAA type I or II. [29] Also, all persons in this study had normal external-ear morphology, a finding that is consistent with the observation that the majority of 18q deletion individuals either have normal external ears or show only minor abnormalities such as low-set ears or prominent helices.

On the basis of our observation that intragenic mutations in *TSHZ1* could only be detected in the four individuals with isolated CAA type IIA who, except for their narrow or atretic auditory canals, have no other congenital malformations, it might be speculated that hemizygosity of *TSHZ1* is limited to and specific to this type of CAA.

The fact that the nonsense mutation in *TSHZ1* in individual 5 was inherited from his phenotypically normal mother can be explained by reduced penetrance. This observation is in accordance with previous reports that describe a CAA incidence of 26% in individuals with an 18q deletion of any kind and an incidence of up to 78% in individuals with a deletion of the critical CAA region that includes *TSHZ1*. [12,16,30] Therefore, nonpenetrance is not unexpected in a carrier of a mutation in the CAA gene.

In conclusion, we have detected both point mutations and copy-number variants leading to haploinsufficiency due to hemizygosity of *TSHZ1* as causes of bilateral CAA type II (in the absence of microtia or anotia) both in isolated nonsyndromic individuals and in persons with the 18q deletion syndrome. Our results provide compelling evidence that the 18q deletion syndrome is a true contiguous gene syndrome, in which the CAA endophenotype is explained by the deletion of *TSHZ1*. Detailed genotype-phenotype studies might further delineate the other phenotypic components of this syndrome.

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chapter 6

# Discussion and future directions

In this thesis, I have explored the implementation of whole genome microarrays in the diagnostic setting and the implications this can have for the generation of phenotypic maps of human chromosomes. On doing so it soon became apparent that the benefits of high resolution genotyping can only be maximally exploited if the process of phenotyping is performed in a comparably sophisticated way. Moreover, in order to create reliable phenotypic maps a critical mass of individuals with overlapping chromosomal aberrations are needed.

While most of the chromosomal anomalies are extremely rare, reliable phenotypic maps may still become within reach by using public databases and internet resources. At the same time, the world-wide web can be used to disseminate the valuable data that will become available by the both quantitatively and qualitatively growing efforts of genotyping and phenotyping, as we showed by the introduction of ECARUCA (Chapter 2).

## Phenotypic maps

The creation of phenotypic maps for rare chromosome aberrations has been actively pursued for over 30 years. One of the first examples is a phenotypic map drawn by Niebuhr in 1978. [1] He searched for the common deleted region on the p-arm of chromosome 5 in 35 patients with microcephaly, a high-pitched cry and mental retardation and was thereby able to create a first map for the Cri du Chat syndrome, concluding that the midportion of the 5p15 segment probably must be deleted in order to develop the main clinical features of this syndrome. [1,2]

Today, we still use the basic principle of these phenotypic maps in order to determine critical regions for clinical signs and dysmorphic features. However, the introduction of new molecular cytogenetic techniques enables us to make much more precise representations of genotype-phenotype correlations. Instead of depicting a relatively large region of several chromosome bands on the p-arm of chromosome 5, Zhang *et al.* were able to delimit the various sub-regions within 5p15.1-p15.33 that are responsible for specific clinical features of the Cri du Chat syndrome. [3] In the same way, we and others applied array CGH results in a group of patients with an 18q deletion (Chapter 4.1). [4,5] Using this approach, critical regions for various clinical features including microcephaly, short stature, white matter disorders and delayed myelination of the brain, kidney malformations and CAA were defined or narrowed.

By getting as much details as possible from the clinical point of view and combining this with high resolution genotyping, we will be able to create highly detailed phenotypic maps containing critical regions of several Mb. [3,5-7] Depending on the number of genes within these

regions, a candidate gene can then be selected and mutation analysis can be performed in new patients with a similar phenotype or an isolated malformation.

This concept has been used throughout genetic history and its effectiveness has been proven by several studies. [8] The introduction of molecular cytogenetic techniques has significantly increased the number of known microdeletion syndromes and single-gene disorders described. [9-11] Among the first and most common clinically recognizable syndromes to arise from widespread testing by FISH of subtelomere deletions was the 9q34 deletion syndrome, now called Kleefstra syndrome. [12,13] Patients show severe intellectual disability, childhood hypotonia with speech and gross motor delay and recognizable facial features. Widespread screening lead to the identification of smaller deletions from 3 Mb to 700 kb. [14-17] Subsequently, Kleefstra and colleagues showed disruption of the *EHMT1* gene in a female patient with a balanced translocation and the 9q34 phenotype. [18] Based on *de novo* point mutations in two patients, they concluded that the 9q34 deletion syndrome is caused by haplo-insufficiency of *EHMT1*, a gene whose protein product (Eu-HMTase1) is a histone H3 Lys 9 (H3-K9) methyltransferase. [18,19]

The traditional sequence of identification of phenotypically similar patients (the syndrome) and the subsequent exploration of its molecular causes, is nowadays often reversed by the identification of novel, recurrent imbalances after screening large numbers of mentally retarded patients by high-resolution techniques. [20] One of the new syndromes identified in this way is the 17q21.31 microdeletion syndrome. [21-23] Only after its molecular identification by high-resolution arrays a specific clinical phenotype was recognized by the investigators.

Recently, it has been shown that this chromosomal syndrome is caused by haploinsufficiency of a single gene, *KANSL1*. [24]

Generating phenotypic maps based on high resolution whole genome techniques can also show the way to genes which, when disrupted, lead to an isolated feature of a particular microdeletion syndrome. This is shown in Chapter 5.2, where we describe the localization and identification of the gene underlying CAA, through the discovery of two small overlapping interstitial microdeletions in individuals with CAA and other features suggestive of an 18q deletion. The minimal region of deletion overlap (72.9-73.4 Mb) contained only one known gene, *TSHZ1*, which was recently shown to be important for murine middle-ear development. [25] Sequence analysis of the coding exons in *TSHZ1* in a cohort of 11 individuals with isolated, nonsyndromic bilateral CAA revealed a loss of function mutation in two individuals. From these results, we concluded that congenital aural atresia is caused by hemizygosity of *TSHZ1* as a result of haploinsufficiency.

The combination of high-resolution molecular cytogenetics with the use of sophisticated phenotypic maps will help clinical geneticists to explain what the future might hold to the parents of a newborn child with a rare chromosome aberration. Furthermore, with each new patient available in a database, the phenotypic map of a specific chromosome disorder becomes more precise. The presence or absence of specific symptoms could lead to a change in size of critical regions or give insight into the penetrance of the feature involved.

At present, microarrays (either oligonucleotide arrays, or SNP arrays) provide the best method we have in order to refine chromosomal regions and sometimes single genes for well-known syndromes. [26-28] When creating phenotypic maps, a pitfall could be that some patients with the exact same deletion or mutation display different phenotypes. Variation in clinical expressivity between patients is the rule, and not the exception. This is shown for example by the variability of congenital aural atresia in patients with either a deletion of the 18q critical region or a *TSHZ1* loss of function mutation. In accordance with previous reports that describe a CAA incidence of 26% to 78% in individuals with an 18q deletion, we detected a nonsense mutation of *TSHZ1* in a phenotypically normal mother of an affected boy (Chapter 5.2). [5,29,30]

It is known that clinical variability can be caused by stochastic factors, epigenetic mechanisms and by the fact that several loci or modifier genes as well as environmental factors can play a role in the expression of clinical features.

Moreover, as it is known that syndromes or isolated malformations that display similar phenotypes can be caused by different genes, other candidate genes should be identified and sequenced in patients without any copy number changes or mutations detected thus far. The upcoming field of bioinformatics will play an important role in the analysis of phenotype data collected within databases, since it can be used to make predictions about new genes for diseases that form part of the same phenotype cluster. This is done by starting from the first gene identified and then searching for genes that are functionally related in gene expression pattern, coevolution, or gene ontology. [31]

It should also be kept in mind that mutations in a new gene are often found in patients with the most severe or typical presentation of the syndrome, i.e. mutations in *TCF4* in Pitt-Hopkins syndrome. [32-34] By investigating more patients, it can become clear that the phenotype is broader than at first expected. In the example of Pitt-Hopkins syndrome, we performed a study of a girl with mild to moderate mental retardation and minor facial anomalies carrying a *de novo* balanced translocation t(18;20)(q21.1;q11.2). As shown in Chapter 5.1, *TCF4* is

disrupted by the translocation breakpoint. We concluded that *TCF4* gene haploinsufficiency can give rise to a much milder clinical phenotype than the previously reported severe mental retardation encephalopathy characteristic of Pitt-Hopkins syndrome and that the possibility exists that many more patients with a milder phenotype carry a mutation in this gene.

An example of the expanding phenotypic spectrum is that of CHARGE syndrome, caused by mutations in *CDH7*. [35] Since the discovery of the involvement of *CDH7* in CHARGE syndrome, numerous unique mutations have been identified, including missense, nonsense and splicing mutations. [36-38] Until today, most of the clinical variability is not due to type or location of the mutation as demonstrated by the differences in clinical presentation in sib pairs with identical mutations. [39] Here, too, a milder phenotype linked to a specific missense mutation has been shown to exist. [40]

## Technique

Now that we have entered the era of cytogenetic molecular techniques and the possibilities for detecting ever-smaller deletions and duplications are almost endless, we may ask ourselves how these new techniques can be used within the field of clinical genetic diagnosis.

With the introduction of ultra-high density genome-wide oligonucleotide- and SNP based microarrays, alterations in the human genome down to 1-10 kb can be detected. This not only leads to a significant added value in detecting chromosome aberrations, but also raises a number of technical and clinical questions, concerning the clinical indications for performing microarray analysis and the genotype-phenotype correlations.

Best practice guidelines and comprehensive genetic counselling have been advocated for accurate correlation and interpretation of the results. [41,42] In order to determine whether a detected deletion or duplication is indeed responsible for the clinical features in a particular patient, a number of aspects need to be checked.



First, DNA of the parents of the patient is investigated in order to check for a *de novo* occurrence. If the aberration is inherited from a normal, healthy parent, it might be less likely to be causal for ID and/or MCA. In this case, the clinician should carefully (re)examine the parent involved in order to look for any subtle dysmorphic signs and/or congenital abnormalities. Due to variable expression and incomplete penetrance, the phenotype of the parent can differ from that of the child. It is hypothesized that the manifestation of some chromosomal imbalances depends on genetic and environmental background, meaning that

familial variants may not always be harmless as was previously assumed. [43] Examples of this phenomenon are seen in individuals with the same 22q13.3 terminal duplication, the 15q13 microdeletion syndrome and deletions/duplications of 16p13.1. [44-46]

On the other side, a *de novo* occurrence is usually taken to be pathogenic. Recent studies have demonstrated that up to 12% of the human genome contains copy number variants (CNVs) that probably not cause any clinical features. [47] The frequency of *de novo* large-segment copy number polymorphisms has been discussed by Van Ommen in 2005. [48] Based on the data for the DMD gene, he estimated that one in every 8 newborns carries a *de novo* segmental deletion and one in every 50 newborns a segmental duplication. Therefore, the finding of a *de novo* alteration in a patient may not be sufficient to draw conclusions about pathogenicity. A major focus of future research will be on the clinical significance of CNVs that are detected in almost every patient sample investigated by high-resolution arrays. The complexity of this area is high and poses a challenge for researchers within the field of medical genetics.

The use of high resolution microarrays and next generation sequencing techniques will provide more insight in the underlying mechanisms of chromosomal rearrangements. In Chapter 3.1 we demonstrate that 46% of karyotypically balanced translocations are actually unbalanced at the submicroscopic level. A recent study using sequencing showed that many such unbalanced translocations involve complex rearrangements with multiple small gains, losses and inversions. [49] This indicates a catastrophic local event similar to the chromothripsis that has been revealed to occur frequently in cancer cells. [50] Chromothripsis has also been demonstrated in germline rearrangements by others. [51] For truly balanced translocations at the sequence level, there appears to be an increase in microhomology of a few bases at the breakpoints. [49] Nonrecurrent CNVs have also been shown to have frequent microhomology at the breakpoints. [52] Finally, there is evidence that recurrent translocations also are driven by local genomic architecture, notably by larger segmental duplications and LCRs. [53]

Secondly, when an aberration is detected, one needs to know whether this alteration has been described in healthy controls, suggesting a non-causative genomic variation. A number of Internet databases can be used, such as the Human Genome Variation Database (http://www.hgvbase.org/) and the Database of Genomic Variants (http://projects.tcag.ca/variation/). [54] However, the finding of microdeletions in healthy humans does not exclude the possibility of pathogenicity. This has been illustrated by Klopocki and colleagues, who detected a common 200 kb microdeletion of chromosome 1q21.1 in patients with Thrombocytopenia-Absent Radius (TAR) syndrome. [55] Although this deletion occurred *de novo* in 25% of patients, 75%

of patients inherited the microdeletion from one of the unaffected parents. Deletions or duplications in this region are not described as CNVs in the Database of Genomic Variants, nor were they detected in 700 other individuals. This resulted in speculations that some microdeletion related phenotypes only develop in the presence of additional modifiers. Recently, Albers and colleagues provided the missing link in TAR etiology. [56] They identified the deficiency of Y14 protein, encoded by the *RBM8A* gene located on the other allele, as the underlying abnormality in TAR. A similar mechanism as the cause of variable expressivity of the 16p12.1 microdeletion has been described by Girirajan and colleagues. [57] These authors describe a second-hit model in which the 16p12.1 microdeletion both acts as a risk factor to neuropsychiatric phenotypes as a single event and exacerbates neurodevelopmental phenotypes in association with large deletions or duplications elsewhere in the genome.

Thirdly, one can explore the medical literature and databases like ECARUCA (www.ecaruca.net) and Decipher (http://decipher.sanger.ac.uk/) whether other patients with the same aberration and a similar phenotype are known. As described in Chapter 2, we launched ECARUCA, a web-based genotype-phenotype database for rare chromosomal aberrations, which is accessible for everyone working in this field. ECARUCA is interactive, dynamic, and has possibilities to store cytogenetic, molecular, and clinical data for the long term. Currently, it contains more than 6,200, mainly unique, chromosomal aberrations detected by routine cytogenetic analysis, FISH, MLPA, and/or genome-wide array analysis in over 4,500 patients. ECARUCA aims to be a database that is easily accessible for all account holders and it encourages both exchanges of information and technical knowledge. It aims to improve patient care and collaboration between genetic centres in the field of clinical cytogenetics.

This free online database grew out to become one of the largest genetic registries with curated genetic and clinical information in the world.



Currently, ECARUCA is collaborating with Cartagenia BENCH (www.cartagenia.com), a software and database platform which aims to automate the entire routine diagnostic laboratory flow. It allows the laboratory to build its internal database, while at the same time external registries are embedded. It has a strong clinical focus and will facilitate ECARUCA by simplifying the submission of genetic and clinical data from a local database by just a single "mouse click".

Ultimately, database collections such as ECARUCA could serve as an intermediate biobank for DNA samples of patients with rare chromosome disorders and, even more relevant, samples

of undiagnosed patients. DNA samples should be collected and stored in the genetic centre of the patient and in case of relevant studies, DNA can be sent to the research group involved.

At the present time, arrays are still relatively expensive and require substantial laboratory expertise. Nonetheless, the costs per experiment have lowered significantly over a short period and the cost of performing whole-genome arrays is currently less than that of standard G-banded karyotyping using microscopic methods. The rapid progress in the development of cheaper commercial whole-genome arrays, coupled with the realisation that most chromosome abnormalities are unique, leaves no doubt that whole genome approaches are the method of first choice in postnatal cytogenetics of patients with intellectual disability and/or congenital malformations.

Although we might sometimes forget, financial feasibility and expertise are not as easily available in all laboratories around the world, especially in less-developed countries. Therefore I would like to argue for laboratories performing whole-genome microarrays as a service to those with limited access, thereby guaranteeing a constant quality and correctness of interpretation of the test results. Clinical genetic centres that lack the ability to investigate patients with these revolutionary techniques should be able to submit samples to the specialized laboratory in order to have equal access.

## From karyotyping to microarray in prenatal diagnosis

Now that high resolution whole genome approaches are the method of first choice in postnatal cytogenetics, one could discuss on the application of these techniques in prenatal diagnosis. Most prenatal cytogenetic laboratories perform conventional karyotyping, thereby detecting abnormalities in 9% to 27% of pregnancies in which ultrasound abnormalities have been observed. [58-60] For most of these anomalies the phenotype is known.

The rate at which array CGH can detect clinically significant submicroscopic aberrations in fetuses with ultrasound anomalies ranges from 2% to 16% in retrospective studies. [61-63] However, although array analysis allows for a much higher resolution genome-wide scan of unbalanced genomic aberrations, its prospective application in prenatal diagnosis is under debate, in particular because of concerns about uninterpretable CNVs and the associated parental anxiety.

In Chapter 3.2, we presented the outcome of a prospective study evaluating several aspects of a new strategy in routine prenatal diagnosis; replacing karyotyping by non-targeted,

whole genome 250K SNP array analysis in fetuses with structural US anomalies. In our experience, most future parents prefer the high resolution application, even though this can lead to unsolicited findings. Despite the low frequency (2%) of clinically relevant submicroscopic aberrations detected, we do promote the use of non-targeted whole genome array analysis in this group of fetuses, as it provides a more accurate and reliable whole genome scan within the same time frame as karyotyping. Recently, Lee and colleagues tested the clinical utility of whole genome microarray in a large cohort of 3171 fetuses. [64] In line with our opinion, they concluded that prenatal array CGH is a valuable tool for screening for chromosomal microdeletions and -duplications, particularly in fetuses with normal karyotypes and structural ultrasound anomalies and should be included as a routine prenatal screening tool.

Clinical guidelines can be of help in the future integration of new molecular techniques in the daily workflow of prenatal diagnosis. [65]

Furthermore, Lee *et al*. recommend array CGH analysis in all fetuses with a *de novo* chromosome anomaly, including apparently balanced translocations. [64] The frequency of congenital abnormalities in fetuses and newborns with *de novo*, reciprocal translocations or inversions has been estimated at 6.1 and 9.4%, respectively. [66] The combined results of previous reports and our study presented in Chapter 3.1 has shown that in 46% of patients with a *de novo* apparently balanced chromosome rearrangement and developmental delay and/or congenital anomalies, a small genomic imbalance could be detected. The chance of finding an imbalance is highest in patients with a complex chromosome rearrangement and/or a De Vries score of at least 3. [67] The use of a similar clinical checklist might be of use in the prenatal setting as well, thereby improving the diagnostic pick up rate of genomic imbalances among fetuses with ultrasound anomalies.



## Phenotyping

In contrast to the enormous improvement of techniques to investigate a person's genotype, the development of instruments to determine a phenotype remains behind. Or better said; the use of new techniques in this field did not find its way to implementation yet. How can this be explained?

At present, the common manner for the establishment of a phenotype is a description of physical features, subdivided in dysmorphic traits, malformations and disorders of organs and

organ functions. Describing dysmorphic features is largely subjective and much depends on individual recognition: the more often you see a feature, the more easily you will recognize it. Standardized definitions and terms to describe the physical variations used in human phenotypic analyses were developed during a project called the *Elements of Morphology*. Almost 400 phenotypic variations of the head and face; periorbital region; ear, nose, and philtrum; mouth and lips; and hands and feet have been defined and terminology for the trunk, genital region skin and remainder of the limb will follow. [68-74]

As an objective support, a computer program for dysmorphology using 3D images has been developed (http://www.3dmd.com/) and can currently be used for, amongst others, Williams syndrome and Wolf-Hirschhorn syndrome. [75,76] One can imagine that this technique can be helpful to clinicians in making a diagnosis in other chromosome disorders and syndromes in which patients display a common pattern of facial features.

This technique has potential utility for Fabry disease, a lysosomal storage disorder. Although facial dysmorphisms are not considered a prominent sign in this disorder, computerized 3D images revealed significant differences in face shape. [77] However, since the discrimination between patients and healthy controls is too low, this new technique will probably not play a key role in the diagnostic recognition of Fabry disease.

For syndromes in which patients display more prominent features, such as Cornelia de Lange, Noonan syndrome or specific chromosome aberrations, the discrimination level of 3D images is higher. Yet, in such genetic disorders a clinician will have a higher chance of making the correct diagnosis from the facial features as well and the added value of computerized phenotyping is not yet significant. Perhaps the 3D images can play a role in clinical training in order to precisely point out the prominent and subtle features in different syndromes to clinical geneticists in training. [76] Furthermore, these computerized techniques can contribute to genotype-phenotype mapping once a cytogenetic aberration or gene mutation has been established, as shown by a milder facial WHS-phenotype in individuals with a small terminal deletion. [75]

Nowadays the behavioural phenotype is more and more appreciated as an important aspect of the phenotype in patients with syndromes and rare chromosome aberrations. So far, behavioural aspects of the phenotype are often poorly investigated and documented in rare chromosome aberrations. However, behavioural studies are gaining popularity among clinical scientists, mainly because of the hypothesis that behaviour is to a large degree based in genetics. Different groups are investigating neurobehavioural phenotypes in various (chromosomal)

syndromes, like Smith-Magenis and Fragile X syndrome. [78,79] In patients with chromosome disorders, specific forms of behaviour can be explicitly present, like sleeping disorders and self-destructive behaviour in Smith-Magenis syndrome, laughing paroxysms in Angelman syndrome and hypersensitivity to sound and high sociability in Williams-Beuren syndrome. [80-82] Most patients carrying a chromosome abnormality have a developmental delay and many have autistic features, aggressiveness or self-injurious behaviour.

More subtle aspects of behaviour like shyness or fondness of specific materials, may also contribute to syndrome recognition. The complex field of studying behavioural genetics will be a challenge for the coming years. The development of complex neuroimaging techniques for the in-vivo scanning of activity in the human brain, like functional Magnetic Resonance Imaging (fMRI) and Magnetoencephalography (MEG), allows researchers to analyse brain function in detail. [83] This technique can offer new possibilities to gain insight in the aetiology of the specific behavioural profile of syndromes, as has been shown in patients with Williams syndrome. [84-86] These latter studies revealed evidence of a different neurofunctional processing of music and noise stimuli in patients with Williams syndrome compared to healthy individuals. Furthermore, a widely distributed network in (sub)cortical structures is activated during music processing in WS patients, whereas this is not seen in healthy individuals. In studies using mouse knockout models, similarities have been revealed between neural systems in patients with Williams syndrome and neuropathological, -physiological and behavioural abnormalities in mice. [87]

The understanding of the aetiology and characteristics of behavioural disorders is not only interesting from a biological point of view, but may be similarly important for parents and other caretakers.

In Chapter 4.2 we describe the neuropsychiatric features of a patient with a proximally located interstitial 18q deletion. The limited literature on proximal interstitial 18q deletions does not allow constructing a concise psychopathological phenotype. However, extensive investigation of this patient showed that the clinical picture is mainly characterised by symptoms from the motor (impulsivity, distractibility and disinhibition) and motivation (dysexecutive signs) domains. These clinical characteristics exclude a treatment with antidepressants or mood stabilisers and indicate the utility of behavioural measures that can be combined with symptomatic psycho-pharmacotherapy.

The knowledge that a specific behaviour is part of the phenotype that is caused by the chromosome disorder, could make it easier to understand and cope with these matters.

Moreover, insight in the aetiology of behaviour could ultimately lead to the design of rational and effective interventions to handle this complex aspect of chromosomal aberration syndromes.

With the advent of technologies that allow fast whole genome sequencing, computerized phenotyping and Internet-based databases, one may assume that all these developments are going to have a significant impact on the care for the individual patient.

However, making the diagnosis can be one of the most challenging tasks for medical doctors, and this is especially the case for rare diseases. In a survey of eight relatively *common* rare diseases such as Marfan syndrome, it was found that 25% of patients waited from 5 to 30 years for a diagnosis and that the initial diagnosis was wrong in 40% of the cases. [88] Although exact statistics are not available, it is safe to believe that the situation is even worse for most of the other, rarer diseases.

In the group of individuals with intellectual disability and/or congenital anomalies the introduction of high resolution whole genome analysis lead to an increase of diagnoses in 10-15%. [89-92] The recent introduction of next generation sequencing techniques is leading to the identification of new Mendelian disease genes in many well known syndromes as well as in idiopathic intellectual disability. [93-102]

Nevertheless, in a significant percentage of patients the cause of the handicap remains unidentified.

Major clinical problems result from delayed or inaccurate diagnosis including delayed treatment, unnecessary diagnostic procedures, and a psychological burden on patients and families because of persistent uncertainty about the cause and prognosis of their clinical problems. Studies have shown that certainty about a diagnosis in children with developmental delay leads to psychological benefit for their mothers. [103] Therefore, the increase in the percentage of the causative factor in patients with previously unexplained intellectual disability is of great value.

Furthermore, increasing awareness among medical doctors and parents on the possible occurrence of specific organ malformations due to a rare chromosome aberration is of great importance for careful screening and improved healthcare (Chapter 4.3).

## Patient empowerment and patient organisation networks

ECARUCA is primarily a database providing information on rare unbalanced chromosome aberrations to professionals. Why did we specifically choose to provide all information to care-providers of patients with chromosomal imbalances and their family members? Because an increase in knowledge on these rare aberrations will lead to a higher quality of personal counselling by the clinical geneticist. Most parents crave for digital documentation and we do feel that there also should be information available on the Internet that is written for non-professionals. Therefore we support the initiative of Unique, a rare chromosome disorder support group, http://www.rarechromo.org, which provides leaflets on over 100 rare chromosome aberrations. These information leaflets are written by professionals and are family-friendly, medically-verified and disorder-specific.

Another organisation that provides freely available information on rare diseases is Orphanet, http://orpha.net. This organisation provides services for patients, professionals, support groups, industry and also for the general public and the information is provided in six different languages. Presently, the information on a lot of rare chromosome disorders is under construction.

The Dutch organisation for rare chromosome aberrations is Zeldzaam, http://www.zeldzame-syndromen.nl/. Their website includes brochures on a small number of chromosome disorders which have been published in the newsletter of the patient organisation.

The virtual umbrella organisation of European support groups for people with rare chromosome disorders is named Eurochromnet and can be found at http://www.eurochromnet.org.

A more general umbrella organisation concerning rare diseases is The European Organisation for Rare Diseases, Eurordis (http://www.eurordis.org/), a patient-driven alliance of patient organisations and individuals active in the field of rare diseases. Eurordis' mission is to build a strong pan-European community of patient organisations and people living with rare diseases, to be their voice at the European level, and - directly or indirectly - to fight against the impact of rare diseases on their lives.

The European Union Science and Society Work Programme for 2006 was focused on the idea that both policy makers and citizens should be equipped to make informed choices from the ever-growing range of options thrown up by scientific and technological progress. This is very true for European rare disease patients and patient organisations, who often wish to take part in research activities on their disease, but don't know where to start or

simply do not master the language used in research. A project called CAPOIRA, funded by the European Commission and leaded by The European Organisation for Rare Diseases, Eurordis (http://www.eurordis.org/), was set up to respond to these needs. The main idea is to foster the participation of patient organisations in research activities by increasing their knowledge, skills and capabilities.

A close collaboration between European patient support groups and professional organisations can be a firm ground to perform scientific research on one hand and returning information on relevant results to patients on the other hand.

In the Netherlands follow-up of patients with rare chromosome disorders takes place in two centres in a multi-disciplinary setting, in Groningen and Nijmegen. Through the exchange of information between parents and medical doctors that occurs within these out-patient clinics, a constant increase in genotype-phenotype knowledge and patient empowerment takes place.

## Concluding remarks

In summary, it is clear that careful phenotyping using standardized approaches, the storage of such information in public accessible databases and bioinformatic skills will be of paramount importance if we are to translate the enormous potential for genotyping of individuals that is before us, into meaningful knowledge for doctors, patients and families.

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# Summary
# Samenvatting

## SUMMARY

An ever increasing number of chromosomal syndromes has been identified, since the first microscopic visualization of human chromosomes. Chromosomal rearrangements are an important cause of distinctive and recognizable clinical phenotypes, with considerable clinical variation between patients. Relatively common cytogenetic syndromes like Cri du Chat and Wolf-Hirschhorn syndrome were studied extensively, and by the late 1970's first attempts were made to construct phenotypic maps of the critical regions for these chromosomal syndromes. An historical overview of the deconstruction of a number of chromosomal aberrations and the establishment of the critical regions of specific features in a phenotypic map is described in ***Chapter 1***.

A considerable improvement in diagnostic techniques in recent years has led to the identification of many new submicroscopic chromosome aberrations. At the same time, the use of new molecular techniques enabled detailed breakpoint mapping in known, microscopically visible chromosomal syndromes. For some conditions, the phenotype appears to be primarily caused by disruption of a single gene, whereas other cytogenetic syndromes are a contiguous gene deletion syndrome.

Abnormal phenotypes have played significant roles in the discovery of critical chromosome regions and gene function, and the organized collection of phenotype data has had trouble keeping up with the developments in genetic technology. Standardized phenotype nomenclature is crucial for a 'Human Phenome Project', in which comprehensive databases are created for such systematically collected clinical information. Some advances were made in the field of computational recognition of a facial phenotype by using 3D imaging techniques, but this technology is not yet in standard use and requires further improvement.

Due to the considerable improvement in diagnostic techniques, more and smaller chromosomal aberrations are detected. Accurate clinical knowledge about rare chromosome disorders is frequently lacking, mostly due to a significant decline in published case reports. At the same time, there is an increasing demand from parents and physicians for reliable prognostic information. In ***Chapter 2***, we describe a genotype-phenotype database for rare, unbalanced chromosomal aberrations named ECARUCA. This web-based database is accessible for everyone working in this field, and aims to increase the level of knowledge among physicians, patients, and their families.

The ECARUCA database contains cytogenetic and clinical data of patients with rare chromosome abnormalities, including microscopically visible aberrations, as well as microdeletions

and -duplications. The ECARUCA database provides health care workers with accurate information on clinical aspects of rare chromosome disorders. ECARUCA is dynamic and professionals submit new cases regularly. Frequent submission of new data ensures the up-to-date quality of the collection. From a scientific perspective, detailed correlations between chromosome aberrations and their phenotypes may aid in deconstructing chromosomal syndromes and localising genes responsible for specific features.

In ***Chapter 3.1***, we studied submicroscopic imbalances in individuals with congenital abnormalities or developmental delay and an apparently balanced, *de novo* translocation or inversion.
We added the results of genome-wide array analysis in 54 patients to data on 117 patients from the literature. A chromosome imbalance was detected in 37% of all patients with two-breakpoint rearrangements. In 49% of these patients, the imbalances were located in one or both breakpoint regions. Imbalances were more frequently (90%) found in complex rearrangements, with the majority (81%) having deletions in the breakpoint regions. We applied a predictive clinical scoring system, the adjusted De Vries criteria, that indicates the complexity of the phenotype. The median De Vries score was significantly higher in those patients with an imbalance compared to patients with a normal array result.
This study provided accurate percentages of cryptic imbalances that can be detected by genome-wide array analysis in simple and complex de novo microscopically balanced chromosome rearrangements and confirmed that these imbalances are more likely to occur in patients with a complex phenotype.

In ***Chapter 3.2***, the use of non-targeted whole genome array analysis in a prenatal diagnostic setting is described. Upon the detection of structural fetal anomalies, parents were counseled and were offered a choice between quantitative fluorescence (QF)-PCR followed by a 250K single-nucleotide polymorphism (SNP)array analysis (QF/array) or QF-PCR followed by a routine karyotyping (QF/karyo). The majority of parents (70%) choose for the QF/array analysis. First, a QF-PCR was performed, which detected an abnormal result in 23% of the fetuses. The remaining samples were analyzed by array, which revealed clinically relevant aberrations, including two known microdeletions, in 4.2% of the cases. Inherited copy number variants (CNV) were detected in 9.3% of the fetuses and CNVs with uncertain clinical relevance in 2.5%.
In 30% of the cases, QF/karyo was requested. Of these, 34% of the samples were abnormal with QF-PCR, and in 6.8% of the remaining cases an abnormal karyotype was found. Two of these were classified as causally related to the ultrasound abnormalities.

This study showed that QF/array does not reveal a high percentage of (sub)microscopic aberrations in fetuses with unselected structural anomalies. However, microarray analysis is preferred over QF/karyo, as it provides a whole genome scan at high resolution, without additional tests needed. The chance of detecting a chromosome aberration not related to the ultrasound anomalies appears to be low.

In ***Chapter 4.1***, the phenotype associated with chromosome 18q deletions is discussed. DNA samples of 29 individuals with an 18q deletion were investigated by a tiling resolution chromosome 18 array to determine the exact breakpoints. Subsequently, we linked the genotype to the patient's phenotype and integrated our results with those previously published. Using this approach, we were able to refine the critical regions for microcephaly (18q21.33), short stature (18q12.1-q12.3, 18q21.1-q21.33 and 18q22.3-q23), white matter disorders and delayed myelination (18q22.3-q23), growth hormone insufficiency (18q22.3-q23) and congenital aural atresia (18q22.3).
The overall level of intellectual disability appeared to be mild in patients with deletions distal to 18q21.33 and severe in patients with deletions proximal to 18q21.31. The critical region for the 'typical' 18q- phenotype was determined to be a region of 4.3 Mb located within 18q22.3-q23.
This study allowed us to update the phenotypic map for chromosome 18q deletions.

In ***Chapter 4.2***, we report on the neuropsychiatric phenotype in a patient with an 18q12.1q22.1 deletion.
His behavioural phenotype was characterized by a non-specific profile of behaviours, the absence of expressive language and poorly developed receptive verbal comprehension as well as by a desire for physical contact in the absence of insight, self-appraisal and differentiated mental processes. Only a small number of individuals with a proximally located 18q deletion have been described and the neuropsychiatric phenotype is not well documented. It includes disorganized and disinhibited behaviours as well as language difficulties. Non development of language seems to be specific for individuals with a more proximally located interstitial 18q deletion.
Although the literature on 18q deletions did not allow constructing a precise psychopathological phenotype, it is of importance that symptoms from the affective, anxiety and psychotic domains were not described in previous case reports. The clinical picture is mainly characterised by symptoms from the motor (impulsivity, distractibility and disinhibition) and motivation (dysexecutive signs) domains. These clinical characteristics exclude a treatment with antidepressants or mood stabilisers and instead indicate the utility of behavioural measures.

In ***Chapter 4.3***, the occurrence of various forms of cardiac anomalies in 19 individuals with a *de novo* 18qter deletion was studied. The heart problems in these individuals varied from relatively common pulmonary valve anomalies to very rare cardiac malformations like Ebstein anomaly in two individuals. All 19 individuals shared a small overlapping deletion region between 18q22.3qter.
The finding of Ebstein anomaly in two 18q deletion individuals suggested the presence of one or more genes within this chromosome region involved in the etiology of this rare cardiac defect. Among the genes located within the critical region was a highly interesting candidate gene named Nuclear Factor for Activated T-Cells (*NFATC1*), a member of the Rel/NF-kb family of transcription factors, which have been implicated in different aspects of embryonic development and transcriptional regulation. Our study supported the findings that *NFATC1* plays an important role in human cardiac development and we suggested that disruption of this gene can lead to Ebstein anomaly. Additional molecularly based genotype-phenotype studies are needed to unravel the exact genotype-phenotype correlation.
This study points out the importance of a careful cardiac evaluation consisting of physical examination, ECG and ultrasound examination in all individuals diagnosed with the 18q deletion syndrome.

In ***Chapter 5.1***, we describe a girl with a *de novo* balanced translocation t(18;20)(q21.1;q11.2). She had mild to moderate intellectual disability and minor facial anomalies.
Breakpoint-mapping by fluorescence in situ hybridization indicated that the basic helix-loop-helix transcription factor *TCF4* gene was disrupted by the breakpoint on chromosome 18. *TCF4* plays a role in cell fate determination and differentiation and mutations in this gene have been shown to result in Pitt-Hopkins syndrome (PHS), characterized by severe ID, epilepsy, mild growth retardation, microcephaly and daily bouts of hyperventilation starting in infancy. Furthermore, patients show distinctive facial features including deep-set eyes, broad nasal bridge, and wide mouth with widely spaced teeth.
Breakpoint mapping on the derivative chromosome 20 indicated that here the rearrangement disrupted the chromodomain helicase DNA binding protein 6 (*CHD6*) gene. To date, there is no indication that *CHD6* is involved in disease.
Our study indicated that *TCF4* gene mutations are not always associated with classical PHS but can give rise to a much milder clinical phenotype.

In ***Chapter 5.2***, the discovery of the genetic cause of congenital aural atresia (CAA) type 2A is described.

CAA can occur as an isolated congenital malformation or in the context of a number of monogenic and chromosomal syndromes. CAA is frequently seen in individuals with an 18q deletion. Four individuals with CAA and other features suggestive of an 18q deletion were studied and overlapping microdeletions in 18q22.3 were detected. The minimal region of overlap between these deletions contained only one known gene, *TSHZ1*, which has been shown to be important for murine middle-ear development.
Sequence analysis of the coding exons in *TSHZ1* in a cohort of 11 individuals with isolated, nonsyndromic bilateral CAA subsequently revealed loss of function mutations in four individuals.

In ***Chapter 6***, the importance of developing standardized, reliable phenotyping in relation to the advances made in molecular cytogenetic techniques, and sharing of this information in public databases, is discussed. With submission of the genotype and the phenotypic information of each new patient in a database, the phenotypic map of a specific chromosome disorder becomes more precise and may further add to disease gene discovery. However, in contrast to the enormous improvement of techniques to investigate a person's genotype, the development of instruments to determine a phenotype lags behind. Yet, the precise delineation of a rare chromosome aberration is not what patients or their parents are looking for. What they in fact want to know, is what the future consequences are likely to be. Combining high-resolution genotyping and sophisticated phenotypic maps in interactive, open-access databases is crucial if we are to translate the enormous potential for genotyping of individuals that is before us, into meaningful knowledge for doctors, patients and families.

## SAMENVATTING

Vanaf het moment dat het mogelijk werd de menselijke chromosomen door een microscoop te bestuderen zijn een toenemend aantal chromosomale syndromen geïdentificeerd. Veranderingen in zowel aantal als vorm van de chromosomen zijn een belangrijke oorzaak van verschillende herkenbare chromosomale syndromen, waarbij vrijwel altijd sprake is van een aanzienlijke variatie tussen patiënten. Relatief vaak voorkomende cytogenetische afwijkingen zoals het Cri du Chat en het Wolf-Hirschhorn syndroom, zijn intensief bestudeerd en aan het einde van de jaren '70 van de vorige eeuw zijn de eerste pogingen gedaan om fenotype overzichtskaarten te maken. Hierbij wordt bepaald welke chromosomale regio's (het genotype) een rol spelen bij het ontstaan van de specifieke klinische kenmerken (het fenotype). Dergelijke chromosomale gebieden die gerelateerd zijn aan een specifiek klinisch kenmerk worden kritische regio's genoemd. Een historisch overzicht van een aantal chromosoomafwijkingen en de bijbehorende fenotype overzichtskaarten wordt beschreven in ***Hoofdstuk 1***. Recente verbeteringen in diagnostische technieken, waarbij kleinere afwijkingen opgespoord kunnen worden, hebben ertoe geleid dat veel nieuwe submicroscopische chromosoomafwijkingen zijn geïdentificeerd. Het gebruik van deze nieuwe moleculaire technieken heeft het tegelijkertijd mogelijk gemaakt om de breukpunten van reeds bekende, microscopisch zichtbare chromosoomafwijkingen nauwkeuriger in kaart te brengen. Bij een aantal syndromen blijkt het fenotype bepaald te worden door de afwezigheid van één enkel gen, terwijl andere beelden juist het gevolg zijn van het tegelijkertijd afwezig zijn van een aantal genen in de kritische regio van het chromosoom.

Het zorgvuldig bestuderen van afwijkende fenotypes heeft een belangrijke rol gespeeld bij het in kaart brengen van de kritische regio's voor de fenotypes, evenals bij het bepalen van de functie van genen. De vooruitgang in het gestructureerd verzamelen van fenotypische informatie verloopt langzamer dan de ontwikkeling van genetische technieken. Om een zogenaamd "Humaan Fenotype Project" te laten slagen, een project waarbij databases worden ontwikkeld voor de systematische opslag van klinische gegevens, is het invoeren van fenotype nomenclatuur cruciaal. Er zijn stappen gemaakt op het gebied van biometrische gezichtsherkenning van een aantal syndromen, waarbij gebruik wordt gemaakt van 3D beeldvormende technieken. Echter, deze technologie maakt op dit moment nog geen onderdeel uit van het standaard klinisch genetisch onderzoek en dient verder geoptimaliseerd te worden.

Door de significante verbeteringen op het gebied van genetische diagnostiek, worden steeds meer en kleinere chromosoomveranderingen gevonden. De kennis over de klinische gevolgen

van zeldzame chromosoomafwijkingen is meestal beperkt, onder andere ten gevolge van een sterke daling in de publicaties van individuele case reports. Tegelijkertijd hebben ouders en dokters behoefte aan betrouwbare informatie. In ***Hoofdstuk 2*** beschrijven we ECARUCA, een genotype-fenotype database voor zeldzame, ongebalanceerde chromosoomafwijkingen. Deze via internet toegankelijke database kan gebruikt worden door iedereen die werkzaam is op het gebied van zeldzame chromosoomafwijkingen en heeft als doel om de kennis bij artsen, patiënten en families te vergroten.
De ECARUCA database bevat cytogenetische en klinische informatie over personen met een zeldzame chromosoomafwijking, zowel microscopisch zichtbare afwijkingen als microdeleties en -duplicaties. ECARUCA voorziet artsen van betrouwbare informatie aangaande klinische afwijkingen die voor kunnen komen bij zeldzame chromosoomafwijkingen. Gebruikers voeren tevens informatie over de bij hen bekende patiënten in, wat ECARUCA interactief, dynamisch en up-to-date maakt. Vanuit wetenschappelijk oogpunt is het delen van genotype-fenotype informatie eveneens waardevol, omdat dit leidt tot het verkleinen van kritische regio's en kan helpen bij het opsporen van nieuwe kandidaatgenen.

In ***Hoofdstuk 3.1*** wordt het onderzoek beschreven bij personen met een ontwikkelingsachterstand of aangeboren afwijkingen, bij wie een microscopisch bepaalde, ogenschijnlijk gebalanceerde, *de novo* chromosoomverandering, zoals een translocatie of een inversie, was vastgesteld. Bij 54 personen werd microarray onderzoek verricht om een submicroscopische deletie of duplicatie op te sporen en de data van 117 personen beschreven in de medische literatuur werd hieraan toegevoegd. Bij 37% van alle personen met een chromosoomverandering waarbij sprake was van twee breukpunten, werd een microdeletie of -duplicatie gevonden. In 49% van deze gevallen bevond de deletie of duplicatie zich rondom een of beide breukpunten. Bij personen met een complexe chromosoomverandering (≥3 breukpunten) werd in 90% van de gevallen een ongebalanceerde afwijking gevonden, waarbij meestal sprake was van een deletie (81%). Om de complexiteit van het fenotype aan te geven, hebben we gebruik gemaakt van een aangepaste versie van de De Vries criteria, een systeem om klinische kenmerken te scoren. De gemiddelde De Vries score was significant hoger bij personen met een ongebalanceerde chromosoomafwijking in vergelijking met de personen waarbij de uitslag van het microarray onderzoek normaal was.
Dit onderzoek geeft een accuraat overzicht van de kans op een submicroscopische afwijking bij personen met een *de novo*, ogenschijnlijk gebalanceerde chromosoomverandering en bevestigt dat microdeleties en -duplicaties vaker voorkomen bij personen met een complex fenotype.

In ***Hoofdstuk 3.2*** wordt het gebruik van genoombreed microarray-onderzoek in de prenatale diagnostiek beschreven. Wanneer tijdens een zwangerschap structurele echoscopische afwijkingen werden vastgesteld, werden de zwangere en haar partner gecounseld over de mogelijkheden van genetisch onderzoek. Paren konden kiezen voor quantitatieve fluorescentie (QF)-PCR, gevolgd door een 250K microarray onderzoek (QF/array), of voor QF-PCR gevolgd door karyotypering (QF/karyo). De meerderheid van de ouders (70%) koos voor de optie QF/array onderzoek. Als eerste werd een QF-PCR onderzoek verricht, waarbij in 23% van de foetussen een afwijking werd gevonden. Bij de resterende monsters werd vervolgens een microarray-onderzoek verricht, waarbij in 4.2% van de gevallen een klinisch relevante afwijking werd gevonden. In 33% van het totaal aantal array-afwijkingen was sprake van een microdeletie, welke niet met karyotypering zou zijn gevonden. Parentaal overgeërfde CNVs werden bij 9.3% van de foetussen gevonden en bij 2.5% was sprake van een CNV met onbekende klinische relevantie.
In 30% van de gevallen werd de optie QF/karyo gekozen. Bij 34% van de ontvangen monsters was sprake van een afwijkende QF-PCR uitslag en in 6.8% van de overige gevallen werd een afwijkend karyogram gezien.
Dit onderzoek toonde aan dat het gebruik van QF/array onderzoek niet leidt tot een hoger percentage vastgestelde (sub)microscopische afwijkingen bij foetussen met algemene structurele echoscopische afwijkingen. Toch wordt de voorkeur gegeven aan de microarray-analyse, omdat het gebruik van deze genoombrede, hoog-resolutie techniek de voorheen gebruikte aanvullende onderzoeken na microscopische karyotypering overbodig maakt. De kans dat met microarray-analyse een (klinisch relevante) chromosoomafwijking wordt gevonden die geen relatie heeft met de echoscopische afwijkingen lijkt in deze studie laag te zijn.

In ***Hoofdstuk 4.1*** wordt het fenotype van personen met een 18q deletie beschreven. Om de exacte breukpunten te bepalen, werden DNA-monsters van 29 personen met een 18q deletie onderzocht met behulp van een hoog-resolutie chromosoom 18 array. Vervolgens werd het genotype van iedere persoon gekoppeld aan het fenotype en tot slot gecombineerd met gegevens uit de medische literatuur.
Op deze manier was het mogelijk om de kritische regio's voor diverse klinische kenmerken in kaart te brengen; microcefalie (18q21.33), kleine lengte (18q12.1-q12.3, 18q21.1-q21.33 en 18q22.3-q23), witte stof-afwijkingen en vertraagde myelinisatie (18q22.3-q23), groeihormoondeficiëntie (18q22.3-q23) en congenitale gehoorgangatresie (18q22.3). Bij personen met een distaal gelokaliseerde deletie (vanaf breukpunt 18q21.33) was over het algemeen sprake van een milde verstandelijke beperking, terwijl deze ernstig was bij personen met een

deletie proximaal van 18q21.31. De kritische regio voor het 18q deletie syndroom fenotype, bestaande uit de combinatie van gehoorgangatresie, voetafwijkingen, een aantal uiterlijke gezichtskenmerken en een milde ontwikkelingsachterstand, werd gelokaliseerd in een gebied van 4.3 Mb in 18q22.3q23.
Door dit onderzoek kon een update van de fenotype overzichtskaart van chromosoom 18q worden gemaakt.

In ***Hoofdstuk 4.2*** beschrijven we het neuropsychiatrische fenotype van een persoon met een 18q12.1q22.1 deletie. Zijn gedrag werd gekenmerkt door een niet-specifiek profiel, de afwezigheid van spraak, onderontwikkeling van verbaal begrip en een sterke behoefte aan fysiek contact. Er was geen of onvoldoende sprake van zelfinzicht en -kennis, alsmede van gedifferentieerde mentale processen. Er zijn slechts een klein aantal personen met een proximaal gelokaliseerde 18q deletie beschreven in de medische literatuur en over het neuropsychiatrische fenotype is weinig bekend. Kenmerken die er deel van uitmaken zijn ongestructureerd en ongeremd gedrag, evenals taalproblemen. Het niet ontwikkelen van gesproken taal lijkt specifiek te zijn voor personen met een proximaal gelokaliseerde interstitiële 18q deletie.
Het was niet mogelijk om een gedetailleerde overzichtskaart te construeren voor het psychopathologische fenotype, maar er worden geen personen beschreven met afwijkingen van de affectieve, angst-, of psychotische domeinen. Het klinisch beeld wordt met name gekenmerkt door symptomen van het motore domein (impulsiviteit, afleidbaarheid en ongeremdheid) en het motivatiedomein (onder andere verminderde cognitieve functies). Bij deze personen is een behandeling met antidepressiva of stemmingstabilisatoren gecontraïndiceerd en kan de behandeling beter op het gedrag worden gericht.

In ***Hoofdstuk 4.3*** worden verschillende congenitale hartafwijkingen bij 19 personen met een de novo 18q deletie beschreven. De hartafwijkingen varieerden van relatief frequente afwijkingen van de pulmonalisklep tot zeer zeldzame aanlegstoornissen, zoals een Ebstein anomalie, welke bij twee personen aanwezig was. Bij alle 19 personen was sprake van een terminale 18q deletie en de kritische regio werd gelokaliseerd in 18q22.3qter.
De aanwezigheid van een Ebstein anomalie bij twee personen met een 18q deletie zou erop kunnen wijzen dat zich een of meerdere genen in deze chromosomale regio bevinden welke een rol spelen bij het ontstaan van deze zeldzame hartafwijking. In deze regio ligt een interessant kandidaatgen, *NFATC1*, lid van de Rel/NF-kb-familie van transcriptiefactoren, welke een rol spelen bij diverse aspecten van embryonale ontwikkeling en transcriptieregulatie. Dit onderzoek ondersteunt eerdere publicaties dat *NFATC1* een belangrijke rol speelt bij de ontwikkeling van het humane hart en onze hypothese is dat disruptie van dit gen tot een

Ebstein anomalie kan leiden. Aanvullende genotype-fenotype-onderzoeken zullen nodig zijn om de exacte correlatie tussen *NFATC1* en Ebstein anomalie te achterhalen.
Dit onderzoek toonde aan dat een zorgvuldig cardiologisch onderzoek, bestaande uit een lichamelijk en echoscopisch onderzoek en een ECG, geïndiceerd is bij alle personen met het (terminale) 18q deletie syndroom.

In ***Hoofdstuk 5.1*** beschrijven we een meisje met een *de novo* gebalanceerde translocatie t(18;20)(q21.1;q11.2). Ze had een milde tot matige verstandelijke beperking en enkele faciale dysmorfieën.
Het in kaart brengen van de breukpunten met behulp van FISH toonde aan dat het breukpunt op chromosoom 18 gelokaliseerd was in *TCF4*. *TCF4* speelt een rol bij celdifferentiatie en mutaties in dit gen zijn aangetoond bij personen met het Pitt-Hopkins syndroom (PHS), wat gekenmerkt wordt door een ernstige verstandelijke beperking, epilepsie, milde groeiachterstand, microcefalie en aanvallen van hyperventilatie vanaf de babyleeftijd. Verder hebben personen met PHS een aantal opvallende uiterlijke kenmerken, zoals diepliggende ogen, een brede neusbrug en een brede mond met vergroting van de ruimte tussen de tanden.
Het in kaart brengen van het breukpunt op het derivaatchromosoom 20 toonde aan dat dit leidde tot disruptie van *CHD6*. Er is tot op heden niet bekend of *CHD6* een rol speelt bij het ontstaan van een ziektebeeld.
Dit onderzoek toonde aan dat mutaties in *TCF4* niet altijd geassocieerd zijn met het klassieke PHS, maar ook kunnen leiden tot een milder fenotype.

In ***Hoofdstuk 5.2*** wordt de ontdekking van de genetische oorzaak van congenitale gehoorgangatresie (CAA) type 2A beschreven. CAA is beschreven als geïsoleerde congenitale afwijking, maar het kan ook onderdeel uitmaken van een aantal monogenetische en chromosomale syndromen. CAA wordt vaker gezien bij personen met een 18q deletie. Vier personen met CAA en een aantal andere kenmerken die passen bij een 18q deletie werden onderzocht en hierbij werden overlappende microdeleties in 18q22.3 aangetoond. De kritische regio bevatte slechts één gen, *TSHZ1*, waarvan bekend is dat deze een belangrijke rol speelt bij de ontwikkeling van het middenoor bij muizen. Vervolgens vond sequentieanalyse van *TSHZ1* plaats in een cohort van 11 personen met geïsoleerde, non-syndromale, bilaterale CAA en hierbij werden mutaties in vier personen aangetoond.

In ***Hoofdstuk 6*** wordt de waarde van gestandaardiseerde, betrouwbare fenotypering in combinatie met gedetailleerde genotypering door moleculaire cytogenetische technieken besproken, alsmede het belang van het invoeren en opslaan van deze gecombineerde data in

openbare databases. Door het invoeren van genotypische en fenotypische informatie van iedere nieuwe patiënt wordt de fenotype overzichtskaart van de betreffende chromosoomafwijking nauwkeuriger en draagt daarmee bij aan het in kaart brengen van verantwoordelijke ziektegenen. In tegenstelling tot de enorme ontwikkelingen op het gebied van genotypering blijft de ontwikkeling van instrumenten om het fenotype te beschrijven achter. De exacte bepaling van de grootte van de chromosoomafwijking is echter niet het enige waar patiënten of hun ouders naar op zoek zijn. De belangrijkste vragen die zij hebben zijn wat deze afwijking voor mogelijke gevolgen kan hebben, nu en in de toekomst. Het combineren van het gedetailleerde genotype en verfijnde fenotype overzichtskaarten in interactieve, publieke, beveiligde databases is cruciaal om de enorme hoeveelheid beschikbare data te vertalen naar zinvolle en begrijpelijke informatie voor artsen, patiënten en families.

# Curriculum Vitae
# List of publications

# CURRICULUM VITAE

Ilse Feenstra werd op 12 oktober 1975 geboren te Heemskerk. In 1993 voltooide zij haar VWO-opleiding aan het Bertrand Russell College in Krommenie, waarna zij begon met de studie Geneeskunde aan de Rijks Universiteit Groningen. Ter afsluiting van haar doctoraalfase verbleef zij gedurende 8 maanden in Philadelphia, Verenigde Staten, voor een wetenschappelijk stage bij de afdeling Obstetrie & Gynaecologie van de Universiteit van Pennsylvania. In 1998 begon zijn aan haar co-schappen in het Medisch Spectrum Twente in Enschede, welke werden afgerond met een keuze-co-schap Obstetrie & Gynaecologie bij de Ziekenhuis Groep Twente in Almelo.

Het artsexamen werd in 2000 behaald, waarna zij aansluitend als ANIOS (arts niet in opleiding tot specialist) werkte bij de afdeling Obstetrie & Gynaecologie bij het eerder genoemde ziekenhuis in Almelo. In 2001 maakte zij de overstap naar de afdeling Obstetrie & Gynaecologie van het Universitair Medisch Centrum Nijmegen (UMCN), waar zij werkzaam was als IVF-arts en onderzoek verrichtte naar de genetische oorzaken van mannelijke fertiliteitstoornissen. Hiermee werd de tot dan toe slapende interesse voor genetica gewekt en in 2003 begon zij als Clinical Database Manager op het door de Europese Unie gefinancierde ECARUCA project op de afdeling Genetica van het UMCN. Dit was de start voor het onderzoek waarvan de resultaten zijn beschreven in dit proefschrift (promotoren Prof. Dr. H.G. Brunner en Prof. Dr. C.M.A. van Ravenswaaij-Arts).

Vanaf oktober 2006 was zij in opleiding tot klinisch geneticus bij deze afdeling (opleiders Prof. Dr. B.C.J. Hamel en Prof. Dr. N.V.A.M. van Slobbe-Knoers). In juli 2010 vond de registratie als klinisch geneticus plaats en sindsdien is zij werkzaam als staflid bij de sectie Klinische Genetica van de afdeling Genetica van het UMCN, met als aandachtsgebieden prenatale diagnostiek, otogenetica en de implementatie van nieuwe, genoombrede technieken in de klinisch genetische praktijk.

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